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PHARMACOLOGICAL MANIPULATION OF THE TISSUE DISTRIBUTION OF ARABINOSYLADENINE 5'-MONOPHOSPHATE IN THE MOUSE

by

© KENNETH GORDON PIPPUS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

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To Greg, Sharon, and Mom

Abstract

9-β-D-Arabinofuranosyladenine (araA) is an adenosine-deoxyadenosine analogue with clinical utility in the treatment of DNA viral disease. The 5'-monophosphate derivative of araA, araAMP, is converted to araA *in vivo*. AraAMP is in clinical trial as a prodrug form of araA, with numerous advantages because it is much more water soluble than araA. The present study demonstrated similar tissue distributions of arabinoside metabolites following administration of equimolar doses of araA and araAMP to mice.

The high aqueous solubility of araAMP avails a number of administrative protocols for that drug which are not feasible with the parent nucleoside. We compared tissue distributions resulting from administration of araAMP by intraperitoneal injection, intravenous injection, and intravenous infusion. The results suggest that rapid intravenous administration of araAMP may be therapeutically advantageous.

AraA enters cells via a nucleoside transport mechanism. Inhibition of this transporter by nitrobenzylthioinosine has been shown to alter the tissue distribution of some nucleoside drugs. We demonstrated perturbation of the pharmacokinetics of araAMP by prior treatment of mice with nitrobenzylthioinosine 5'-monophosphate. Effects of such treatment on plasma and spleen metabolites of araAMP were also observed. Data are presented which suggest that transport of arabinonucleosides in the spleen may differ from the conventional understanding of nucleoside transport.

AraA is inactivated *in vivo* by conversion to arabinosylhypoxanthine, a reaction catalyzed by adenosine deaminase. Treatment of mice with deoxycoformycin, a potent inhibitor of adenosine deaminase, was shown to alter the pharmacokinetics and metabolism of ara-AMP.

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List of Abbreviations

ADP	Adenosine 5'-diphosphate
AraA	9-β-D-Arabinofuranosyladenine
AraADP	9-β-D-Arabinofuranosyladenine 5'-diphosphate
AraAMP	9-β-D-Arabinofuranosyladenine 5'-monophosphate
AraATP	9-β-D-Arabinofuranosyladenine 5'-triphosphate
AraC	9-β-D-Arabinofuranosylcytosine
AraH	9-β-D-Arabinofuranosylhypoxanthine
ATP	Adenosine 5'-triphosphate
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine
HNBTGR	(2-Amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine
HSV I	Herpes Simplex Virus Type I
HSV II	Herpes Simplex Virus Type II
IDU	5-Iodo-2'-deoxyuridine
i.p.	Intraperitoneal
i.v.	Intravenous
NBMPR	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosyl purine (nitrobenzylthioinosine)
NBMPR-P	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosyl purine 5'-monophosphate (nitrobenzylthioinosine 5'-monophosphate)

PCA	Perchloric acid
TLC	Thin layer chromatography
Tubercidin	4-Amino-7-(β-D-ribofuranosyl)pyrollo-[2,3 *d*]-pyrimidine
2'-dCF	(R)-3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo-[4,5 *d*]-[1,3]-diazepin-8-ol

I. Introduction

A. Nucleoside Drugs

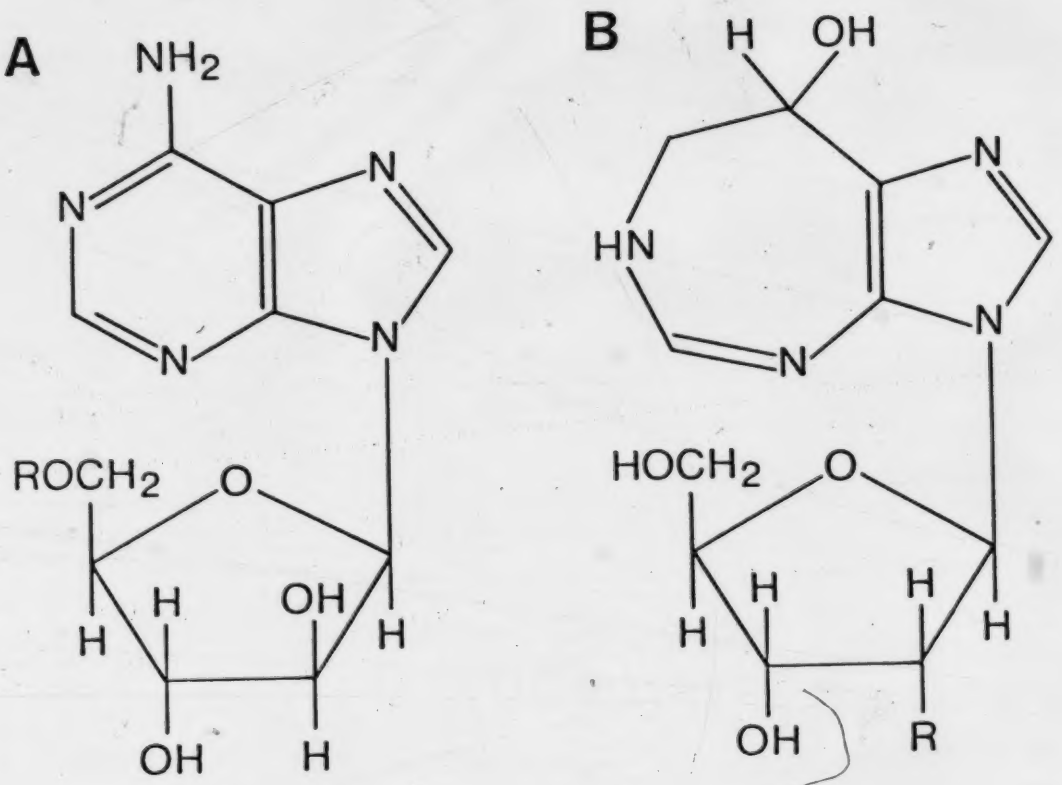
AraA (9-β-D-arabinofuranosyladenine, adenine arabinoside, vidarabine, Figure 1) is an adenosine-deoxyadenosine analogue with antitumor (Brink and LePage, 1964) and antiviral (Sacks et al., 1979) activity. The solubility of araA in water is about 0.5 mg/ml at 25° (Repta et al., 1975). AraAMP (9-β-D-arabinofuranosyladenine 5'-monophosphate, viraMP, Figure 1) is a derivative of araA with high water solublility which is dephosphorylated in vivo (LePage et al., 1972) and which is presently in clinical trial as a "prodrug" form of araA (Whitley et al., 1980).

Other drugs used in this study include nitrobenzylthioinosine (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine, NBMPR) and HNBTGR (2-amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine),both potent inhibitors of nucleoside transport by mammalian cells (Paul et al., 1975), and NBMPR-P, the highly soluble 5'-monophosphate of NBMPR.

Deoxycoformycin ((R)-3-(2-deoxy-β-D-erythro-pentofuranosyl)-3,6,7,8-tetrahydroimidazo-[4,5d]-[1,3]diazepin-8-ol, 2'-dCF, Figure 1) was employed as an inhibitor of adenosine deaminase (Woo et al., 1974).

B. Synthetic and Preclinical History of AraA

AraA was originally synthesized as a potential anticancer drug (Lee et al., 1960). Despite evidence of antiproliferative activity



I, R = H
II, R = PO_3

III, R = OH
IV, R = H

Fig. 1. Chemical structures of arabinoside drugs and adenosine deaminase inhibitors. I: AraA. II: AraAMP. III: Coformycin. IV: Deoxycoformycin.

towards neoplastic cells in culture and some success in therapy of malignant disease in experimental animals (Brink and LePage, 1964a; Brink and LePage, 1965; Doering et al., 1966), araA has not advanced beyond clinical trials as an anti-cancer agent (LePage, 1972). A facile chemical synthesis for the drug was reported (Glandemans and Fletcher, 1963) shortly before the first report of the drug's antiviral activity (Privat de Garilhe and Rudder, 1964). Research was still restricted by limited drug availability until an antibiotic isolated from Streptomyces antibioticus cultures was identified as araA (Parke-Davis and Co., Belgian Patent No. 671,557, 1967).

Since the initial report of the sensitivity of herpes simplex virus type I (HSV I), viruses of several groups have been demonstrated to be sensitive to araA in vitro (for an extensive review see Shannon, 1975) and in vivo (see Sloan, 1975). Antiviral activity in vitro was demonstrated against various DNA viruses (herpesviruses, poxviruses, adenoviruses, and iridoviruses), and against RNA viruses of the rhabdovirus and oncornavirus groups. The rhabdoviruses are the only group so far recognized which exhibit sensitivity to araA and which do not require DNA synthesis for replication. Neither rabies virus nor vesicular stomatitis virus (both are rhabdoviruses) is sensitive to araA in vivo (Janis and Harmon, 1974).

Preclinical investigations have indicated that araA has considerable antiviral activity in animal models of human infection, notably against viruses of the herpes group. The first report of therapeutic activity in the treatment of viral disease in an animal

model described reduction in disease symptoms and an increase in survival following corneal inoculation with HSV I. This effect was achieved by topical application of araA in ophthalmic ointment, and was found to be dose-dependent (Sidwell et al., 1968). That dose dependency probably explained earlier reports that araA was ineffective in the treatment of herpetic keratitis in the rabbit (Johnson and Jervey, 1964); it would seem that dosages in the earlier studies were below an effective range (0.06% solution in H_2O).

When administered by topical application, araA had a therapeutic index of >60 in ocular infection, whereas arabinosylcytosine (araC) and 5-iodo-2'-deoxyuridine (IDU) had therapeutic indices of 2-4 (Sidwell et al., 1968). AraA was effective in the treatment of corneal infections of rabbits and hamsters with HSV I and vaccinia virus when administered topically, subconjunctivally, intraocularly or subcutaneously. Subconjunctival administration was also effective in the therapy of corneal HSV I infection in the owl monkey (Sloan, 1975). AraA was also useful in the treatment of ocular disease involving deeper tissues. Subconjunctival injection of 5% araA suspensions had significant therapeutic effects on herpetic uveitis in rabbits and owl monkeys, even when there was considerable stromal involvement (Kaufman et al., 1970).

The observation that subcutaneous administration of a suspension of araA was effective in controlling ophthalmic disease suggested potential utility in the parenteral therapy of other viral disease. Therapeutic value was demonstrated when it was observed that i.p.

administration of araA following inoculation of hairless mice with HSV I reduced the severity of lesions, and prevented the extensive neurological involvement and lethality which occurred in untreated animals (Klein et al., 1974).

More interesting from a clinical perspective was the observation that i.p. treatment with araA protected mice from the lethal effects of intracerebral infection with either HSV I or vaccinia virus (Schabel, 1968). This was the first instance of success in the treatment of viral encephalitis in an animal model. Peroral treatment with araA was also effective in intracerebrally infected mice, although large (1000 mg/kg twice daily for five days) dosages were required in this instance (Sloan et al., 1968).

These promising results in the therapy of cerebral disease in animals prompted the subsequent clinical trials of the drug in human herpes encephalitis.

C. Clinical History of AraA

The results of clinical trials with araA have largely paralleled the observations made in experimental animal models of viral disease. The initial indications of clinical utility for the drug were seen in a double blind trial against viral keratoconjunctivitis, with IDU as the blind control (Pavan-Langston and Dohlman, 1972). The two agents, administered in ophthalmic ointment, were found to be of roughly equal efficacy in most patients. AraA was, however, effective in disease which was resistant to IDU. Additionally,

healing of deeply ulcerated eyes seemed to progress more rapidly after treatment with araA than with IDU. In an isolated case of vaccinial keratitis, araA treatment produced a dramatic cure, but, like IDU, araA had no apparent effect in ocular disease caused by adenovirus.

The demonstration of efficacy in the treatment of herpetic eye disease was followed by a report describing effective treatment of such disease by parenteral administration of araA (Brightbill and Kaufman, 1974). Following intramuscular injection of araA, therapeutically effective concentrations of araA and araH were found in the aqueous humor of the eye, and healing of viral lesions was observed.

AraA was also effective in therapy of disseminated herpetic disease when the drug was given by intravenous infusion. Progressive mucocutaneous infections with both herpes labialis and herpes genitalia in immunosuppressed patients were well controlled by i.v. infusion of araA (Ch'ien et al., 1973). Inhibition of viral replication and healing of lesions were much slower in patients with HSV II than in HSV I, even when both infections occurred in the same patient. Subsequent studies have described antiviral efficacy in immunosuppressed patients with disseminated infection resulting from herpes zoster virus (Whitley et al., 1975).

AraA was demonstrated to have effect in the treatment of neonatal infection with HSV II. Before the advent of therapy with araA, mortality from this condition approached 50%, with survivors

often suffering severe neurological sequellae. AraA treatment significantly reduced both mortality and the incidence of sequellae in survivors (Ch'ien et al., 1973; Whitley et al., 1975). Early treatment greatly improved the prognosis in this condition. Though HSV II is less sensitive to araA than HSV I in immunosuppressed patients, success in the treatment of neonatal HSV II indicates clinical utility of the drug against that virus.

Perhaps the most dramatic success of parenteral therapy with araA has been in the treatment of encephalitis caused by HSV I. Both araC and IDU were in trial for treatment of this devastating, life-threatening disease (Nolan et al., 1970; Rappel et al., 1971; Farris and Blow, 1972; Chow et al., 1973) before the introduction of araA, but controlled trials indicated that both agents actually had therapeutic indices of less than 1, and treatment with either resulted in increased mortality (Boston Interhospital Study Group, 1975).

The initial double blind comparison of araA and placebo in the treatment of HSV encephalitis provided a remarkable contrast between the therapeutic activity of araA and that of IDU or araC. Treatment with araA reduced mortality from 72% in patients receiving placebo to 30% (Whitley et al., 1977). Therapeutic effects in this study were so convincing that the double blind code was broken very early and the study discontinued, causing some controversy regarding the use of double blind trials of drugs with apparent efficacy in the treatment of life-threatening disease (McCartney, 1978). The wide-

spread use of IDU and araC in herpes encephalitis, despite lack of demonstrable therapeutic effect with either drug, is convincing evidence that such controlled trials are vital to critical evaluation of the utility of a new drug.

In addition to reducing mortality in encephalitis, araA therapy resulted in reduced neurological sequellae in survivors. Prognosis in terms of both mortality and severity of sequellae appeared to be dependent primarily on the degree of neurological involvement at the time treatment was initiated. Since patients still conscious at the time araA was first administered had low mortality rates and moderate or negligible neurological damage, early treatment appeared critical. Although remarkable efficacy, low toxicity and the critical nature of early initiation of treatment would support use of the drug in unconfirmed HSV encephalitis while awaiting biopsy results (Med. Letter, 1979), this approach remains controversial (Braun, 1980).

Several studies (Merigan and Robinson, 1978; Chadwick et al., 1978; Preksaitis et al., 1981) have indicated some promise for araA in the treatment of chronic active hepatitis. Following administration of araA (or araAMP), inhibition of viral replication is observed in most patients, as evidenced by reduction or elimination of Dane particles found in the blood (Merigan and Robinson, 1978). In many patients, however, pretreatment levels of Dane particles were observed again soon after cessation of treatment. The latter observation makes any lasting therapeutic effect doubtful. Optimizing administration parameters, administering araA or araAMP in

combination with inhibitors of catabolism, or combining araA with auxiliary antiviral agents (Sherlock, 1980; Thomas and Bassendine, 1980) may improve the responses obtained in this disease.

The low solubility of araA combines with inherent fluid balance problems in patients with herpetic encephalitis to make fluid overload the limiting drug toxicity in these patients (Whitley et al., 1977). In addition to this problem, the drug exhibits a number of other toxicities. Transient, reversible hepatotoxicity (seen as an elevation of serum glutamic-oxaloacetic transaminase) and myelosuppression have been observed with both araA (Sacks et al., 1979; Keeney, 1975) and araAMP (Whitley et al., 1980; Preksaitis et al., 1981), along with nausea and vomiting. None of these toxicities is dose-limiting.

Myalgias have been observed following araA treatment in the absence of elevated serum muscle enzymes, with normal electromyelogram results and no evidence of compromised peripheral nervous conduction (Sacks et al., 1979). Similarly, various arthralgias and myalgias with no obvious tissue damage at the site of pain have been seen following treatment with araAMP (Preksaitis et al., 1981; Whitley et al., 1980). This neurological toxicity appears to be greatly enhanced by compromised renal function, and also by coadministration of allopurinol (Friedman, 1981). It appears that hepatic and renal function should be assessed at the time of prescription of araA, and closely monitored during treatment with araA (Whitley et al., 1981). The toxicities of araA, though distinctive, do not appear to

limit the utility of the drug in serious viral disease. Its relatively low aqueous solubility does, however, present problems in the treatment of HSV encephalitis.

Two newer agents, acyloguanosine (ACG, 9-(2-hydroxyethoxymethyl)guanine, Elion et al., 1978) and bromovinyluridine (E-5-(2-bromovinyl)-2'-deoxyuridine, DeClerq et al., 1979) are presently in clinical trial. Both agents appear to inhibit HSV replication, with lower host toxicity than araA. In vitro it is not difficult to isolate strains of HSV which are resistant to inhibition by both agents, yet which remain susceptible to araA (Field et al., 1981). This suggests that araA may continue to be a useful antiviral agent when viral strains which are resistant to the more selective actions of these newer drugs become important.

D. Cellular Metabolism of AraA

AraA enters animal cells by facilitated diffusion, as a substrate for the NBMPR-sensitive nucleoside transport mechanism (Cass and Paterson, 1975; Paterson et al., 1981). After entering cells, araA molecules undergo deamination to araH or phosphorylation to araAMP, araADP and araATP (Brink and LePage, 1964).

Since the plasma membrane is impermeable to nucleotides, phosphorylation can effectively trap nucleosides within cells against an apparent concentration gradient (Plunkett and Cohen, 1977b). Deamination of araA results in production of araH, which is not phosphorylated and, therefore, is not trapped within cells (LePage and Brink, 1964).

Deamination of araA is catalyzed by adenosine deaminase (York and LePage, 1966), but araA is a poor substrate for adenosine kinase (Schnebli et al., 1967). The enzymes responsible for phosphorylating araA in mammalian systems are deoxycytidine kinase and deoxyadenosine kinase (Brockman et al., 1980; Krenitsky et al., 1976).

The cytotoxic and antiviral activities of araA evidently result from inhibition of mammalian (Furth and Cohen, 1967; York and LePage, 1966) and HSV-induced (Muller et al., 1977; Muller and Zahn, 1979) DNA polymerase. The herpes virus-induced polymerase is more sensitive to inhibition by araATP (K_i, 0.14 μM) than the mammalian enzyme (K_i, 7.4-7.9 μM).

The fact that the affinity of the virus-induced polymerase for araATP is higher than that of the cellular polymerase appears to be partially responsible for the selective inhibition of growth of virally infected cells. A number of studies (Smith et al., 1978; Schwartz et al., 1976; Drach and Shipman, 1977) have shown that DNA synthesis is inhibited in virally infected cells when concentrations of araA in the extracellular medium are lower than those necessary to inhibit synthesis in uninfected cells. These studies have not excluded the possibility that metabolic trapping of araA may also be enhanced in infected cells.

A different mechanism of action for araATP, suggested by York and LePage (1966), is the inhibition of mammalian ribonucleoside-diphosphate reductase. While inhibition of this enzyme would probably deplete deoxynucleotide pools sufficiently to inhibit DNA syn-

thesis, it is difficult to envision a mechanism by which this effect would be specific for virally infected cells, since there are no reports of a virally induced ribonucleotide reductase. In view of the relatively high concentrations of araATP (30–110 μM) required to reduce by 50% the activity of ribonucleotide reductase (Moore and Cohen, 1967), this effect would seem likely to contribute to the antiproliferative effects of araA only in anticancer regimens, in which large doses of the drug or treatment with an adenosine deaminase inhibitor result in high intracellular concentrations of araATP (Shewach and Plunkett, 1979).

AraA, araAMP and araADP have all been shown to inhibit S-adenosylhomocysteine hydrolase, the enzyme responsible for synthesis and hydrolysis of S-adenosylhomocysteine. The latter compound participates as a methyl donor in a number of transmethylation reactions (Trewyn and Kerr, 1976). K_i values for araA, araAMP and araADP in the inhibition of the hydrolase were 5.0, 110 and 1000 μM, respectively. These values pertain only in the absence of adenine, AMP, ADP, adenosine and S-adenosylhomocysteine, all of which protect the enzyme from inhibition (Helland and Ueland, 1981). Inhibition of methylation reactions likely contributes to the toxicity to lymphoblasts observed following combined administration of araA and 2'-dCF to patients (Hershfield and Kredich, 1981). This inhibition apparently results from high intracellular concentrations of araA (and deoxyadenosine) following inhibition of adenosine deaminase.

Nucleotides of araA have been reported to be incorporated into cellular and viral DNA, with no apparent chain-terminating effect

(Muller and Zahn, 1979; Plunkett and Cohen, 1975). This incorporation may be a factor in the chromosome breaks observed in human leukocytes cultured with araA (Nichols, 1964) and the teratogenic effects of araA in rabbits and rats (Fishart et al., 1975; Kurtz et al., 1977). Incorporation of araA into DNA could contribute to the cytotoxic effects of the drug.

E. Derivatives of AraA and the Use of Adjunct Drugs

Despite its landmark role in antiviral chemotherapy, araA is not without its shortcomings. The drug's very low aqueous solubility necessitates a large volume of infusion, compounding fluid balance problems, notably in herpes encephalitis. In addition, rapid deamination to araH appears to limit potency, perhaps limiting araA's antitumor utility (Brink and LePage, 1964; Preksaitis et al., 1981).

A number of derivatives of araA have been synthesized (see Table I). Some of these derivatives are not substrates for adenosine deaminase and others are more soluble than araA. Several analogues are more potent inhibitors of DNA synthesis than araA. Though this enhanced inhibitory potency may have application in cancer chemotherapy (e.g., 2-fluoro araA, Brockman et al., 1977), increased host toxicity has prevented enhancement of antiviral therapeutic indices (or "selectivity indices", see Drach and Shipman, 1977).

Two derivatives with both enhanced solubility and resistance to adenosine deaminase (neither is a substrate) have shown particular promise as prodrug forms of araA. AraA 5'-formate (Repta et al.,

Table I. Derivatives of AraA

The following are examples of araA derivatives found in the literature. For an extensive review, see Haskell (1977).

Drugs described	Reference
2',3' And 3',5' di-O-acyl araA derivatives	Baker et al., 1979
2-Fluoro AraA	Brockman et al., 1977
2'-Azido-2'-deoxy araA	Cermak-North et al., 1979
Carbocyclic araA derivatives	Lee and Vince, 1980
2'-Amino-2'-deoxy araA	Lee et al., 1979
8-Butylamino araA	Neidle et al., 1979
3',5'-Cyclic araAMP	Sidwell et al., 1973
AraA 5'-valerate	Yu et al., 1980

1975) and araAMP (Furth and Cohen, 1967) both appear to be useful primarily because they are pharmacologically inactive until converted to araA. Initial studies with araAMP in man suggested that, in addition to the advantages provided by its enhanced solubility with respect to the parent compound, araA was dephosphorylated very slowly. Thus, araAMP was thought to act as a long lasting, adenosine deaminase-insensitive source of araA, that would provide prolonged, elevated levels of that nucleoside in plasma (LePage et al., 1972, 1975). The possible benefits of this extended time course appear to have focused attention on this drug, while experimentation with araA 5'-formate has dwindled. Other investigators (Whitley et al., 1980; Tyrrell et al., 1980) have been unable to detect sustained high plasma concentrations of either araA or araAMP following treatment with araAMP. Rapid dephosphorylation and deamination result in araH becoming the only detectable metabolite in plasma within 15 min after drug administration. Thus, similar plasma metabolite time courses are seen following administration of araAMP and araA 5'-formate (Repta et al., 1975).

AraAMP is dephosphorylated by 5'-nucleotidases in a number of tissues and in vivo. Because entrance of araAMP into cells is slow (Plunkett and Cohen, 1977) and appearance of araA in plasma is relatively rapid (Tyrrell et al., 1980), araAMP dephosphorylation is evidently catalyzed by plasma enzymes or ectoenzymes. Rapid dephosphorylation apparently negates protection from deamination in man (Preksaitis et al., 1981).

Studies which compared the toxicities of araA and araAMP in animals (Kurtz et al., 1977) and man (Whitley et al., 1980) have suggested that the acute toxicity of araAMP is not significantly different from that of araA. AraAMP was, however, found to be less teratogenic in animals than araA.

One approach to increasing the potency of araA has been that of administering the drug in combination with an inhibitor of adenosine deaminase, to increase the physiological life of araA. Two deaminase inhibitors which have been used in this connection are 2'-deoxycoformycin (2'-dCF), Woo et al., 1974) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, Schaeffer and Schwender, 1974). Both drugs exhibit synergistic antitumor activity with araA (Plunkett and Cohen, 1977a; LePage et al., 1975), and EHNA has been shown to enhance antiviral efficacy in vivo (Shannon et al., 1980) when administered at low dosage levels. Though combinations of araA (or araAMP) and a deaminase inhibitor show some promise in the treatment of human malignancy (Hershfield et al., 1981; Gray et al., 1981), limitations on the utility of these combinations in antiviral treatment may be suggested by the profound immunosuppression observed following administration of araA and 2'-dCF to animals (Lum et al., 1980). The selective inhibition of viral DNA synthesis with minimal effect on uninfected mammalian cells makes araA an effective antiviral agent (Drach and Shipman, 1978).

F. Objectives of this Study

The ability to manipulate tissue distribution of araAMP (or araA) metabolites may have clinical application. Increasing the drug concentration at a target tissue could yield two beneficial effects, increased antiviral or antitumor activity, and decreased host toxicity. Reduction in toxicity to the host could occur by two mechanisms: reduced drug availability at sites of host toxicity, or reduction of the total dose necessary to achieve the desired therapeutic effect, as a result of increased availability at the site of pathology.

The tissue distribution of araAMP may be altered by 4 means:

1. Alteration of administrative regimen
2. Pharmacological interference with cellular uptake of the drug
3. Pharmacological manipulation of drug metabolism
4. Alteration of the drug form administered

In this project, examples of all 4 means were examined.

The low aqueous solubility of araA necessitates administration by intravenous infusion in large volumes of saline or intramuscular injection of a suspension (Sacks et al., 1979). The solubility of the monophosphate derivative makes a wide variety of administrative protocols feasible (Whitely et al., 1980). In an effort to assess what administrative regimen might be advantageous, the tissue distributions resulting from 4 different treatment protocols are compared.

AraA enters mammalian cells by way of a nucleoside transporter of broad specificity (Cass et al., in press). It seems reasonable that inhibition of this transport system would alter uptake of nucleosides into various tissues, and it has been shown that treatment of mice with NBMPR-P prior to administration of the adenosine analogue, tubercidin, alters the tissue distribution and toxicity of that drug (Kolassa et al., in press). Coadministration of NBMPR-P with nebularine has been shown to enhance the therapeutic effect of the latter in the treatment of murine leukemia L1210, apparently by altering the pharmacokinetics of nebularine (Lynch et al., 1981). NBMPR protected mice from otherwise lethal doses of nebularine. Thus, alteration of the distribution of pharmacologically active nucleosides with NBMPR-P has been demonstrated, as has the therapeutic advantage of such an effect. Therefore, we examined the effects of NBMPR-P pretreatment on araAMP distribution in an effort to discover similar effects on distribution which might be exploitable in combination therapy.

Deamination by adenosine deaminase is the principal pathway of detoxification and inactivation of araA (Brink and LePage, 1964). Inhibition of this enzyme should alter in vivo metabolism of araA and araAMP. Hidaka et al., (1980) have shown alterations in the tissue distribution of araA as a result of inhibition of adenosine deaminase, although the methodology of that study did not permit quantification of drug concentrations. The effects on tissue distribution of araAMP produced by prior treatment with 2'-dCF were examined in the present study.

AraAMP is presently regarded as a soluble, "prodrug" form of araA (Whitely et al., 1980; Tyrrell et al., 1980) despite a dearth of published evidence regarding the comparative metabolism and distribution of the two drugs. The tissue distribution and metabolism of araAMP and araA in the mouse were compared in this study, in an effort to assess the validity of that view.

II. Materials and Methods

A. Chemicals

AraAMP monohydrate used throughout this project was the kind gift of Dr. G.A. LePage of the Cancer Research Unit (McEachern Laboratory), University of Alberta. [2,8-^{3}H]AraAMP and [2,8-^{3}H]-araA were purchased from Moravek Biochemicals (Brea, California), and [carboxy-^{14}C]inulin from ICN Pharmaceuticals (Irvine, California). NBMPR-P and HNBTGR were synthesized in this laboratory (Lynch et al., 1981; Paul et al., 1975). 2'-dCF was supplied by the Division of Cancer Treatment, National Cancer Institute (Bethesda, Maryland). Monophase 40, Permafluor V, and Carbosorb (Packard Instruments, Downer's Grove, Illinois), reagents for operating a Packard Model 306 Sample Oxidizer, were purchased from Academy Instruments (Mississauga, Ontario).

B. Animals

Female B10D2F$_1$ mice (C57BL/10J female X DBA/2J, F$_1$), 20-27 g, were obtained from the Health Sciences Small Animal Program of the University of Alberta.

C. Determination of Drug-Derived ^{3}H in Mouse Tissues

Mice were decapitated and bled onto Parafilm (American Can Company, Greenwich, Connecticut). Samples (100 µl) of blood were immediately spotted onto 30 cm^2 pieces of chromatography paper, which were dried at room temperature. Remaining blood was spun at

12,000 x g for three minutes. Measured volumes of the plasma so obtained were dried on chromatography paper.

Within 5 min after decapitation, mice were dissected and individual tissues were placed on tared chromatography paper rectangles. After fresh weights of the tissue samples were determined, the samples were dried overnight at 100°. Subsequently, dry weights were taken.

Tissue samples and the accompanying papers were compressed into tight pellets which were combusted in a Packard Model 306 Sample Oxidizer. In this instrument, the water of combustion is quantitatively trapped and dispensed into counting vials together with scintillant. Radioisotope content was determined using a Beckman LS3133T or LS7500 liquid scintillation counter (Beckman Instruments, Irvine, California).

To follow the urinary excretion of araAMP and its metabolites, mice were placed in 400 ml beakers on filter paper, which retained urine. The ^{3}H content of urine samples collected in this way was determined by the combustion-scintillation counting procedure.

D. I.V. Infusion Procedure

Mice were anaesthetized with methoxyflurane vapour (Penthrane, Abbott Laboratories Ltd., Montreal, Quebec) and tail veins were cannulated with Intramedic PE-10 Tubing (Clay-Adams, Parsippany, New Jersey) by the procedure of Paul and Dave (1975). A wire mesh splint was taped to the base of the tail, and the cannula tubing and

tail were both taped to this splint at two more distal points. A 7 cm glass tube with an inside diameter of 1 cm was taped in place covering tail, tube and splint, and the distal end of the glass tube was wired to the lid of the cage in which the animal was placed. Infusion medium (0.15 M NaCl containing 0.25 mg/ml [2,8-^{3}H]araAMP) was delivered by a syringe mounted in an infusion pump (Model 940, Harvard Apparatus, Millis, Massachussetts). Tissue levels of drug-derived isotope were determined as described in section C, above.

E. Recovery of Administered Dose

Two methods were utilized to determine the fraction of ^{3}H administered as [2,8-^{3}H]araAMP which could be accounted for 1 hr after drug administration: (a) using a balance sheet approach, the ^{3}H contents of all tissues were summed, assuming the total blood volume to be 6 ml/100 g body weight, and the skeletal muscle mass to be 60% of body weight (Green, 1966). To these values were added values for 1 hr urinary excretion as determined in section C, above. Dividing these sums by the total administered ^{3}H yielded a value for fractional recovery. (b) The other method of estimating total recovery involved measuring the total ^{3}H content of mice. The animal was placed in a tared vessel for 1 hr following administration of labelled araAMP, then killed and homogenized in the same vessel with 18 volumes of water (wt/vol), using a Polytron blender (Kinematica GMBH, Lucerne, Switzerland). Samples (100 µl) of homogenate were applied to chromatography paper, dried and assayed for

^3H content by the combustion-scintillation counting procedure. This yielded a value for total ^3H content of tissues and urine which could be compared to the estimate derived from the balance sheet calculation.

F. Volatile Component Determination

Since the procedures for determining tissue content of araAMP and metabolites (see section E, above) involved drying prior to the combustion assay of ^3H, tissue ^3H contents so obtained did not account for ^3H which might have been in a volatile form. An estimate of volatile ^3H (presumably in the form of ^3H_2O) was made in the following manner: 1 hr after administration of [2,8-^3H]-araAMP, mice were decapitated and bled onto parafilm. Blood was immediately transferred to 1.5 ml microcentrifuge tubes and spun at 4°. Six 50 µl samples of plasma from each mouse were dispensed into scintillation vials, and half of these were immediately mixed with 5 ml of tritisol, a xylene-detergent scintillant (Pande, 1976). The remaining samples were freeze-dried, redissolved in 50 µl of distilled water, and 5 ml of tritisol was added to each. Subtracting the mean value for the ^3H content of freeze-dried samples from that of fresh samples yielded a value for the ^3H_2O content of plasma. Since ^3H_2O distributes very rapidly in body water, the plasma content of ^3H_2O was considered to be representative of body fluids in general. This value was used in estimating ^3H_2O lost during drying of tissue samples in our attempts to account for

radioactivity administered by means of a "balance sheet" (Table 6) and in whole body homogenization experiments.

G. Plasma Metabolite Determination

Following decapitation, animals were bled onto parafilm and blood was drawn into 1 ml syringes containing 0.3 ml of 0.15 M NaCl with 100 μM HNBTGR, 10 μM 2'-dCF, and a known concentration of [carboxy-^{14}C)inulin. The HNBTGR and 2'-dCF were present to stop uptake and metabolism of araA by the cellular components of blood during sample processing. The dilution of labelled inulin which resulted from mixing with the blood sample allowed determination of the plasma volume of that sample.

Blood sample mixtures were centrifuged (3 min, 12,000 x g) and 100 μl samples of the supernatant fractions were spotted on paper for subsequent assay of ^{3}H and ^{14}C by the combustion method. Other 100 μl samples of supernatant were combined with 20 μl of 2 M perchloric acid (PCA). These mixtures were kept on ice with intermittent mixing for fifteen minutes, then centrifuged (1 min, 12,000 x g). The supernatant was removed and neutralized with K_2CO_3. This mixture was centrifuged (1 min, 12,000 x g) to remove precipitated $KClO_4$.

The arabinonucleoside content of the neutralized PCA extracts were determined by a thin layer chromatographic (TLC) method in which extract samples were chromatographed together with carrier nucleosides. Silica gel plates with fluorescent indicator (Type

13181, Eastman Kodak Company, Rochester, New York) were employed and were developed in butanol-acetone-ammonia-water (50:40:3:15, v/v; Kolassa et al., 1972). Carrier areas were scraped from the TLC, suspended in water and mixed with tritisol for assay of ^{3}H by scintillation counting.

Nucleotide metabolites formed from [2,8-^{3}H]araAMP in tissues were determined by chromatographing PCA extracts and measuring ^{3}H which accompanied authentic arabinonucleotide carriers (araATP, Sigma Chemical Co., St. Louis, Missouri). Arabinonucleotides were separated by TLC on PEI-Cellulose plates (Baker-Flex Cellulose PEI, J.T. Baker Chemical Company, Phillipsburg, New Jersey). Chromatograms were developed to (i) 2.5 cm above the origin in 0.5 M sodium formate, pH 3.5, (ii) then to 8.0 cm above the origin in 2 M sodium formate, pH 3.5, and (iii) finally to the end of the 20 cm sheet in 4 M sodium formate, pH 3.5, all without drying between solvent changes. ^{3}H-activity accompanied the arabinonucleotide carriers on thin layer PEI-cellulose chromatograms developed in a two dimensional system which separated arabinonucleotides from ribosides and deoxyribosides (Schwarz and Drach, 1975). The ^{3}H content of PEI-cellulose samples was determined by the combustion-scintillation counting procedure.

H. Spleen and Liver Metabolites of araAMP

Levels of araAMP metabolites in liver and spleen were determined by chromatographic analysis of PCA extracts of these tissues. Mice

were anaesthetized with sodium pentobarbital (70 mg/kg, i.p.) 5 min before sampling. Spleens were exposed via an incision immediately under the rib cage on the left side. Livers were exposed by a 2–4 mm transverse incision below the sternum. Immediately upon exposure, either organ was rapidly frozen using a liquid nitrogen-cooled Wollenberger clamp (Wollenberger et al., 1961).

While under liquid nitrogen, freeze-clamped samples were trimmed free of tissue that was not flattened by the chilled blocks of the Wollenberger clamp. Still under liquid nitrogen, the tissue was ground in a porcelain mortar. Samples of ground tissue were added to tared microcentrifuge tubes containing about 10 parts (wt/vol) of ice cold 0.4 M PCA. The tubes were weighed to determine the weight of tissue extracted. After a 30 min interval at 4° with intermittent mixing, the mixtures were centrifuged. The supernatant fractions were neutralized with K_2CO_3, and the neutralized extracts were subjected to chromatographic analysis using the TLC systems described above.

To evaluate the tissue extraction procedure, ATP/ADP ratios in extracts were determined by high pressure liquid chromatography using a 25 cm Whatman SAX ion exchange column (Whatman Inc., Clifton, New Jersey). The column was eluted isocratically with 0.26 M KH_2PO_4 and 0.5 M KCl, pH 4.5 (Brown, 1970). For the spleen extract analyzed, the ratio of ATP and ADP concentrations was 3.1. Ove et al. (1967) have indicated that inadequate conditions (principally time and temperature) in the extraction procedure can result

in lowering of ATP/ADP ratios. Since the ratio measured in this experiment was within the 2.5–4 range reported for other tissues (Keppler et al., 1970; Schultz and Lowenstein, 1978), it appears that this application of the extraction procedure was sound. This HPLC analysis was conducted by Dr. J.D. Moyer, whose assistance is gratefully acknowledged.

III. Tissue Distribution of AraA and AraAMP

A. Results

1. Tissue Distribution of araAMP Derived ^3H

Before evaluating the various means of influencing tissue distribution of araAMP and its metabolites, it was necessary to establish bases for comparison. For this purpose, and additionally to identify the tissues most important in the uptake and metabolism of the drug, the isotopic content of a number of tissues were determined 60 min after i.v. injection of [2,8-^3H]araAMP. The dosage of araAMP monohydrate used throughout this study, 15 mg/kg body weight (41.1 μmol/kg), was used because similar daily dosages are used in man for the treatment of herpes virus encephalitis (Whitley et al., 1980) and hepatitis B (Preksaitis et al., 1981). The drug was administered as a 1.0 mg/ml solution in 0.15 M NaCl. AraAMP doses (0.3 - 0.4 ml) were injected into tail veins during intervals of about 3 sec.

In describing data from tissue distribution experiments, the terms "araAMP equivalent content", "tissue ^3H content", and "araAMP and its metabolites" are used interchangeably in recognition of the fact that araAMP undergoes extensive metabolism in vivo. Tissue metabolites probably consist of araA, araA nucleotides, and araH.

Under the conditions of the experiment described in Figure 2, all tissues assayed had quantifiable ^3H concentrations; all tissues but brain retained significantly higher ^3H concentrations than did plasma. Particularly high ^3H contents were observed in

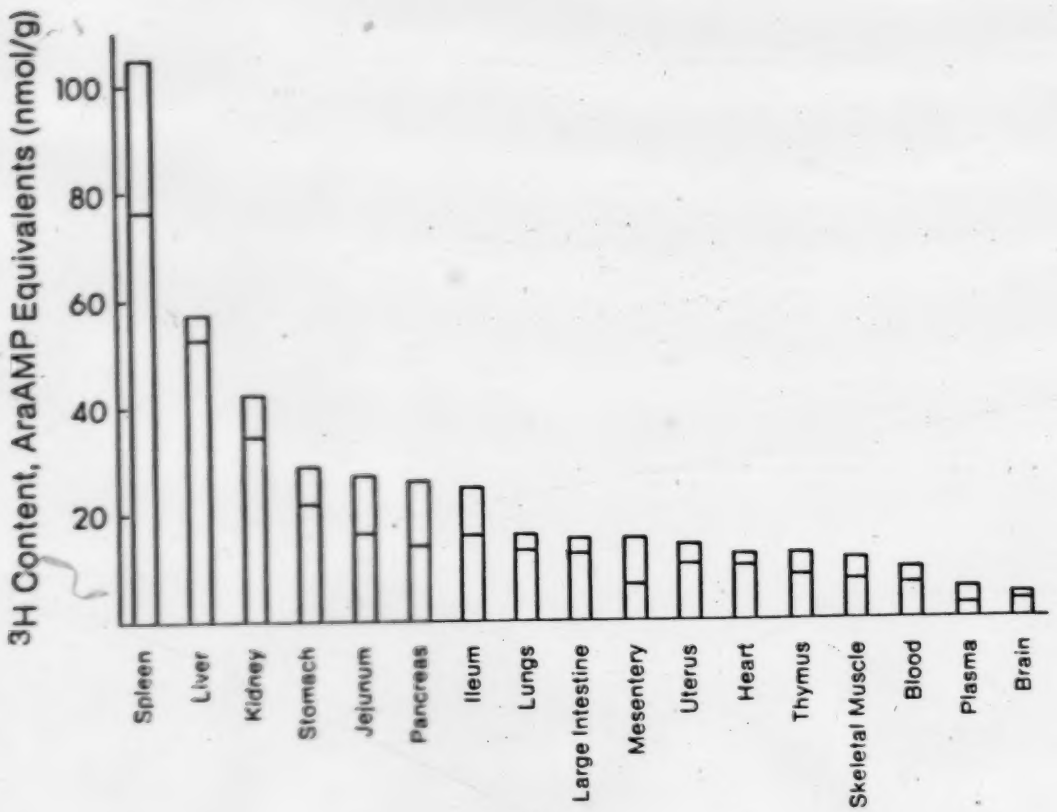


Fig. 2. Distribution of i.v. administered [2,8-^{3}H]araAMP in tissues of the mouse. Female B10D2F$_1$ mice were injected i.v. with 15 mg/kg [2,8]araAMP and tissues were sampled for assay of ^{3}H content 60 min after drug injection. Tissue contents of ^{3}H are expressed as araAMP equivalents. For all tissues, n = 5; the upper bars represent means and the lower bars are 1 standard deviation below the mean.

spleen, liver and kidney. Of the tissues assayed, the araAMP equivalent concentration of spleen was the highest, with a value 19-fold higher than that of plasma. Next highest was liver with a concentration 10-fold higher than plasma, followed by kidney in which the araAMP equivalent concentration of ^{3}H was 7-fold higher than plasma. The lowest araAMP equivalent content observed at this time point for any tissue was that of brain.

The araAMP contents of some mouse tissues measured in the experiment of Figure 2 were compared with values for the same parameter measured 4 hr after AMP administration (Table 2) in order to assess the decline with time in tissue content of araAMP and metabolites. It is seen that tissue concentrations of araAMP metabolites and their distribution between the various tissues measured 4 hr after araAMP injection were similar to those found 3 hr earlier, with the exception of the spleen, where the concentration had fallen considerably since 1 hr after administration. By the 4th hr after araAMP administration, plasma concentrations of ^{3}H had fallen below those of brain, but the ^{3}H contents of liver, spleen and kidney remained much higher than that of plasma.

2. Effects of the Route of Administration on Tissue Distribution of araAMP

In an effort to assess potential advantages of the variety of administrative protocols made possible by the high solubility of araAMP, four administrative regimens were compared with regard to

Table 2. Four hour tissue distribution of i.v. araAMP in the mouse.

Tissues were samples and assayed for ^{3}H content 4 hr after the i.v. administration of 15 mg/kg [2,8-^{3}H]araAMP. Values are expressed as araAMP equivalents of ^{3}H.

Tissue	Metabolite concentration in araAMP equivalents (nmol/g fresh wt ± S.D.)*
Plasma	1.2 ± 0.1
Brain	2.2 ± 0.2
Blood	7.5 ± 1.1
Liver	40.3 ± 3.1
Spleen	43.0 ± 3.8
Kidney	47.2 ± 2.8

*n = 5

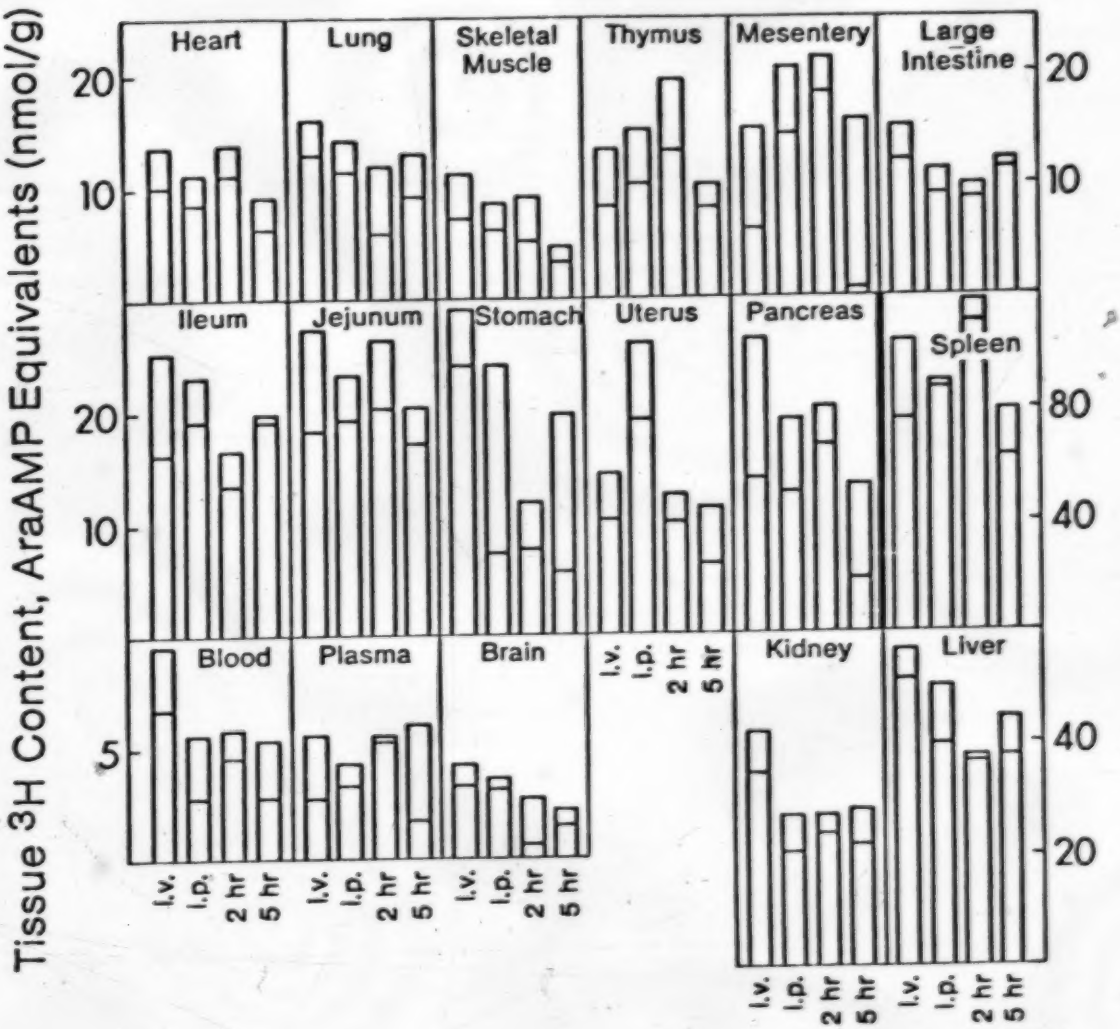


Fig. 3. Effects of administrative protocol on tissue distribution of araAMP. Mice received [2,8-^{3}H]araAMP (15 mg/kg) by (i) i.v. injection over an interval of about 3 sec; (ii) i.p. injection over an interval of about 3 sec; (iii) 2 hr continuous infusion via a tail vein; (iv) 5 hr continuous infusion via a tail vein. Tissues of animals receiving drug by injection were sampled 60 min after injection, those of infused animals were sampled 15 min after completion of infusion. ^{3}H was assayed by the combustion-liquid scintillation counting method. For all tissues, n = 5; upper bars represent sample means and lower bars represent means less 1 standard deviation. Note that the scales for ordinate values for spleen, liver and kidney differ from those for other tissues.

their effects on tissue distribution: i.v. injection, i.p. injection and i.v. infusion of 2 and 5 hr durations. In the experiments of F[illegible] tissues were sampled (a) 60 min after drug injection and (b) 15 min after infusions were ended, because araAMP-derived levels of ^{3}H in plasma were similar at these times for all four administrative regimens.

Uterus was the only tissue in which ^{3}H levels were significantly higher ($p < 0.05$, Student's t-test) after araAMP administration by i.p. injection than after i.v. injection. In other tissues, metabolite concentrations were lower (blood, kidney) or not significantly different following i.p. injection of araAMP from those found after i.v. injection.

No significant enhancement of tissue content of drug relative to that resulting from i.v. injection was seen for either of the i.v. infusion regimens. Lower concentrations ($p < 0.05$, student's t-test) than those observed following i.v. injection were detected in lung, large intestine, stomach, blood, kidney and liver following 2 hr infusion, and in skeletal muscle, blood, brain, kidney and liver following 5 hr infusion.

3. Tissue distribution of araA-derived ^{3}H

In order to evaluate the present view of araAMP as a water-soluble, "prodrug" form of araA, the tissue distribution of araAMP and metabolites was compared with that of the parent nucleoside. The dosage of araA used (11 mg/kg) was the molar equivalent to the dosage of araAMP used throughout this study.

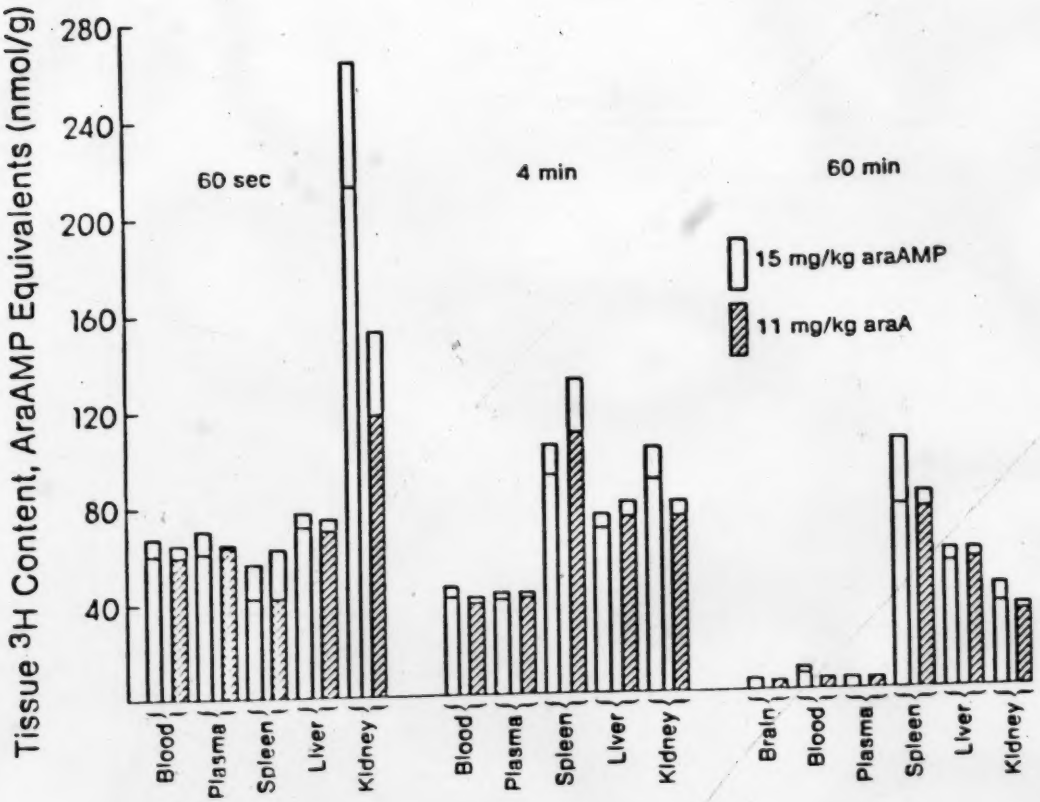


Fig. 4. Comparison of tissue distribution of i.v. administered araAMP with that of araA in the mouse. B10D2F$_1$ female mice were injected with [2,8-^{3}H]araAMP (15 mg/kg, i.v.) or [2,8-^{3}H]araA (11 mg/kg, i.v.). Tissues were sampled for assay of ^{3}H content at the times after injection specified above. For all tissues, n = 5; the upper bars represent sample means and the lower bars represent means less 1 standard deviation.

Tissue distributions of araA-derived ^{3}H were assayed at three time points following i.v. injection and at one after i.p. injection. In the i.v. injection experiments (see Figure 4), drug-equivalent ^{3}H concentrations in the kidney were higher after administration of araAMP than after araA. Isotopic concentrations in other tissues were similar after administration of either the nucleoside or the nucleotide. Although not significant in any individual instance, a general trend towards higher values in tissues other than kidney was found following administration of the nucleotide.

Following i.p. injection (see Figure 5), in contrast, a general trend towards lower isotope levels was seen following administration of araAMP than of araA, except in the spleen. Significantly lower levels are found only in plasma and brain ($p < 0.05$, Student's t-test).

Since liver is a major site of araA and araAMP metabolism, and since drugs administered by the i.p. route enter the hepatic portal circulation before the systemic circulation (Sartorelli and Upchurch, 1963), one might anticipate that i.p. administration of these agents would produce higher levels of drug metabolites in liver than would i.v. administration. As seen in Figure 4, this was not the case; total radiometabolite levels were not significantly different between the two routes of administration 60 min after injection of araAMP.

Since it was thought that dephosphorylation might be the rate-limiting step in liver accumulation of i.p. administered nucleotide (LePage et al., 1972), liver levels arising from i.v. and i.p.

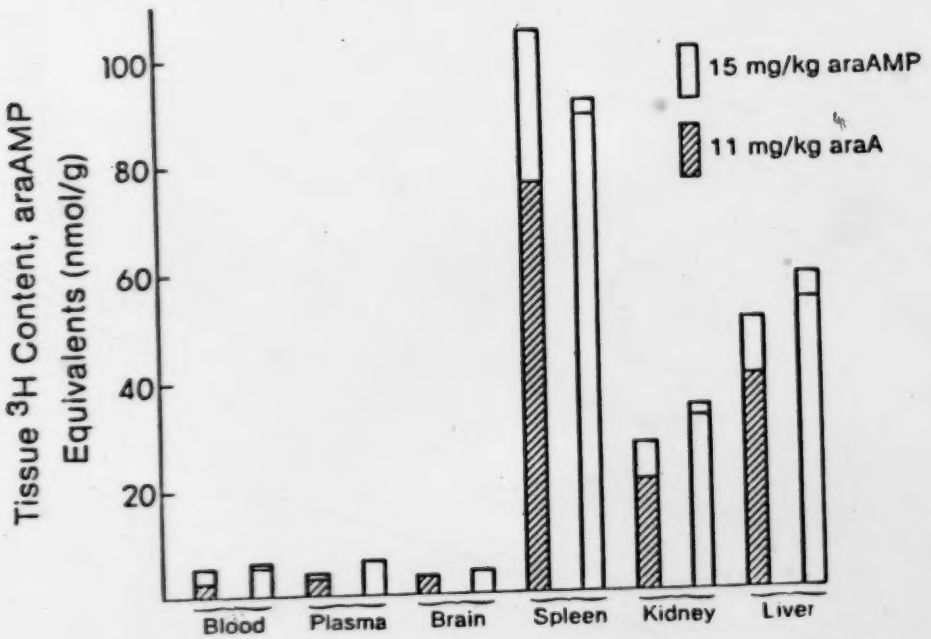


Fig. 5. Tissue distribution of araA and araAMP after i.p. administration. Female B10D2F$_1$ mice received equimolar dosages of either [2,8-^{3}H]araA or [2,8-^{3}H]araAMP by i.p. injection. Tissues were sampled 60 min after injection for assay of ^{3}H content by combustion and liquid scintillation counting. For all tissues, n = 5; the upper bars represent sample means and the lower bars represent means less 1 standard deviation.

Table 3. Liver ^{3}H content following administration of labelled araA or araAMP. Mice received either [2,8-^{3}H]araA (11 mg/kg) or [2,8-^{3}H]araAMP (15 mg/kg) by rapid i.v. or i.p. injection. Livers were sampled 60 min after drug administration for assay of ^{3}H content. For all groups n = 5.

Drug Form Administered	Injection site	Tissue ^{3}H content, araAMP equivalents, nmol/g fresh wt (mean ± S.D.)
araA	i.v.	53.9 ± 4.5
araAMP	i.v.	57.6 ± 5.4
araA	i.p.	58.3 ± 4.2
araAMP	i.p.	50.4 ± 10.5

administration of araA (11 mg/kg), which would not require dephosphorylation to undergo transport, were also compared. As shown in Table 3, the final liver isotopic concentration was remarkably insensitive to the form and route of administration of the drug, with no demonstrable difference between levels achieved following any combination of the two factors.

4. Pharmacological Manipulation of araAMP Tissue Distribution

a. Plasma

Figure 6 illustrates the time course of the nonvolatile ^{3}H content of plasma following the i.v. administration to mice of [2,8-^{3}H]araAMP (15 mg/kg). These data represent the ^{3}H content of dried plasma samples determined by the combustion-scintillation counting method. In animals not treated with NBMPR-P or 2'-dCF, elimination of ^{3}H from plasma followed a complex time course, as is apparent in Figure 6. Isotopic concentrations determined more than 10 min after drug administration appear to decline exponentially; the half life of ^{3}H in plasma determined at these later time points was 19.8 min.

Also presented in Figure 6 is the time course of plasma ^{3}H content which resulted when labelled araAMP was administered 30 min after treatment with NBMPR-P (25 mg/kg, i.p.) or 2'-dCF (10 mg/kg, i.p.). Prior treatment with 2'-dCF had no significant effect on the time course of araAMP-derived ^{3}H in plasma. Prior treatment with the transport inhibitor enhanced elimination of nonvolatile isotope from plasma, reducing the late half life from 19.8 min to 16.4 min.

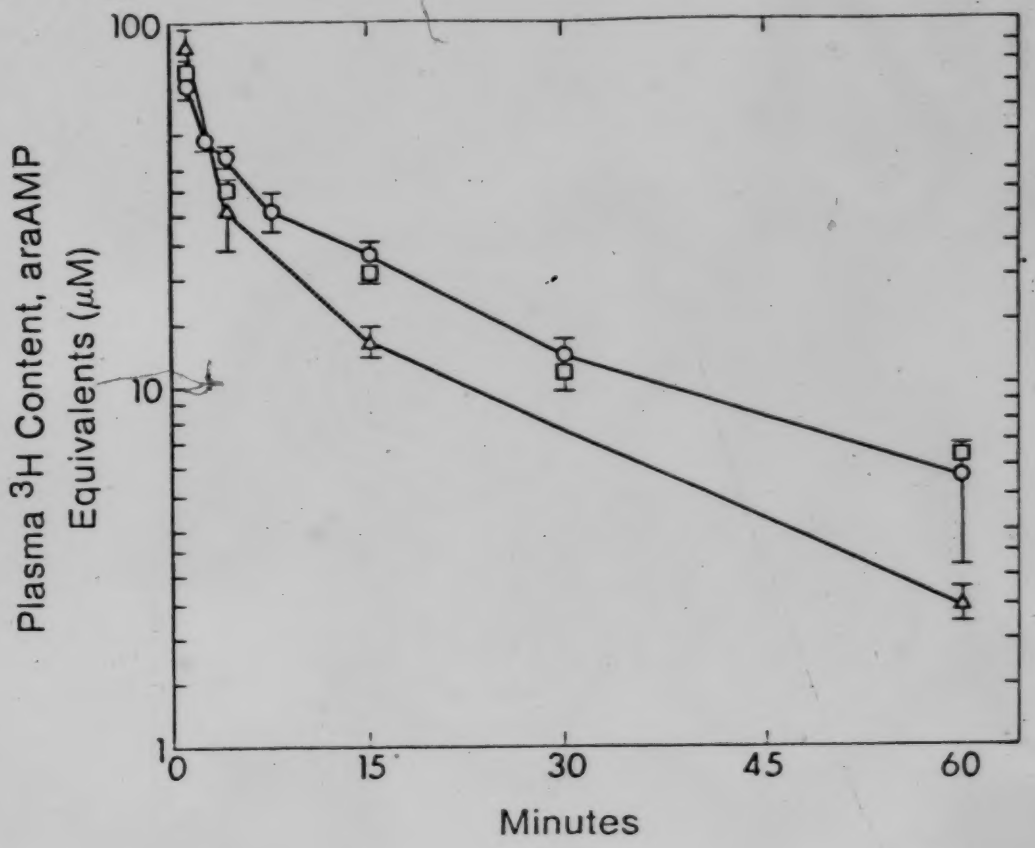


Fig. 6. Time course of araAMP metabolites in plasma. All animals received [2,8-^{3}H]araAMP (15 mg/kg, i.v.). Animals received 2'-dCF (10 mg/kg, i.p.; □), NBMPR-P (25 mg/kg, i.p.; △), or no pretreatment (○) 30 min prior to administration of araAMP. Plasma was assayed for ^{3}H content at the indicated times after araAMP administration by the combustion-scintillation counting method. Data are presented as mean ± S.D., n = 5 for all points.

Table 4. Effect of prior treatment with NBMPR-P on urinary excretion of araAMP-derived ^{3}H.

Female B10D2F$_1$ mice were treated with saline or with NBMPR-P (25 mg/kg, i.p.) 30 min prior to the i.v. administration of [2,8-^{3}H]araAMP (15 mg/kg,). The animals were then kept in a beaker on filter paper for 60 min. This same filter paper was then used to absorb urine excreted following cervical dislocation. The ^{3}H content of the filter paper-collected urine was determined by the combustion-scintillation counting method.

Prior treatment with NBMPR-P mg/kg	Fraction of injected ^{3}H found in urine 60 min after injection (mean ± S.D., n = 5) %
0	46.8 ± 3.1
25	28.7 ± 3.1

While one might expect enhanced clearance from plasma to result from an increased urinary excretion, the experiments summarized in Table 4 showed that prior treatment with NBMPR-P decreased the urinary excretion of ^{3}H derived from araAMP during the first 60-min interval after administration. Thus, though the difference between the plasma half-lives of araA observed in untreated and NBMPR-pretreated animals is of dubious statistical significance, the difference observed is in the opposite direction from that predicted by urinary excretion rate. Accordingly, some other explanation must be sought for the NBMPR-P effect.

b. Liver

In contrast to the exponential decline in plasma concentrations of araAMP which begins (in the practical sense) immediately after injection, the liver content of araAMP rose initially and had attained a maximum concentration within 60 sec. Concentrations declined to 25% of that peak value by 15 min, and remained essentially unchanged from that point until 1 hr, as seen in Figure 7.

NBMPR-P treatment slowed the uptake of araAMP-derived ^{3}H by liver. In mice which had received prior treatment with the transport inhibitor, a peak concentration in liver was not observed until 4 min after administration of araAMP, and values observed at all time points were lower than those found in the untreated animals after the same interval.

Although the isotope contents of the livers of animals treated with 2'-dCF were lower than those of untreated animals 60 sec after administration of araAMP, at 4 min and all subsequent time points,

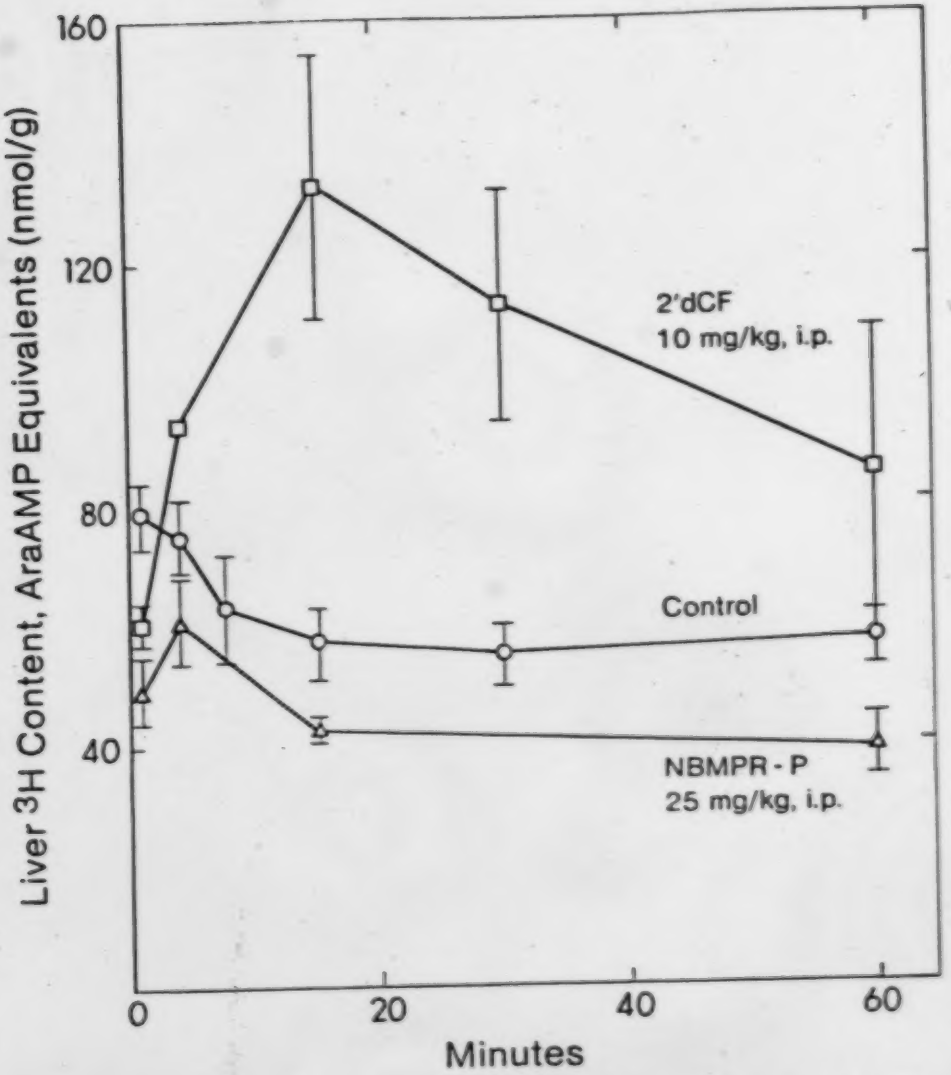


Fig. 7. Pharmacological manipulation of liver accumulation of araAMP. All animals received [2,8-^{3}H]araAMP (15 mg/kg, i.v.). Animals in the pretreated groups received 2'-dCF (10 mg/kg, i.p.) or NBMPR-P (25 mg/kg, i.p.) 30 min prior to administration of araAMP. Livers were removed at the indicated times after araAMP administration for assay of ^{3}H content by the combustion-scintillation counting method. Bars represent mean ± S.D., n = 5 for all points.

2'-dCF treatment resulted in higher values in the liver. The most notable evidence of this enhancement of accumulation was the peak concentration in the 2'-dCF treated animal, which was not attained until 15 min after araAMP administration. At that point, total araA radiometabolites were 2.3-fold more concentrated in livers of the 2'-dCF treated animals than in livers of the control mice.

c. Brain

Maximum isotopic concentrations did not occur in brain until 30 min after administration of labelled araAMP. Of the tissues assayed, the araAMP content in brain was the slowest to reach a peak concentration in the control as well as both pretreated groups of animals. The peak levels observed were also the lowest for any tissue in all three pretreated regimens. As shown in Figure 8, the peak concentration observed in brains of the 2'-dCF treated animals was somewhat higher than that in the control, while NBMPR-P pretreatment resulted in values substantially lower than in the control. This suggests that an NBMPR-P inhibitable transporter mediates the passage of araA through the blood brain barrier.

d. Spleen

Peak isotopic concentrations in the spleen were also observed only at relatively long intervals following drug administration. NBMPR-P-treated, 2'-dCF-treated, and control animals all attained maximum spleen isotope contents 15 min after injection of labelled araAMP (Figure 9). Unlike the results described above, pretreatment with 2'-dCF reduced spleen concentrations of araA metabolites to levels lower than those in control. Conversely, treatment with

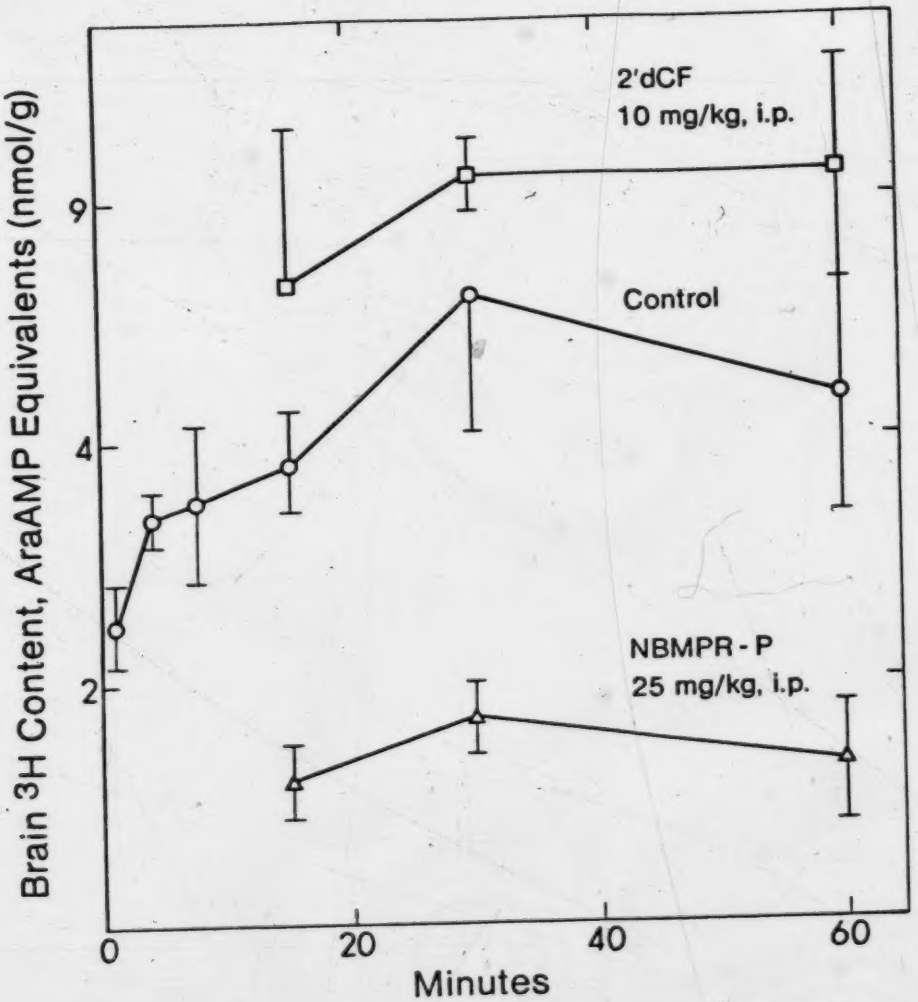


Fig. 8. Accumulation of araAMP metabolites in the brain. All animals received [2,8-^{3}H]araAMP (15 mg/kg, i.v.). Animals in the pretreated groups received 2'-dCF (10 mg/kg, i.p.) or NBMPR-P (25 mg/kg, i.p.) 30 min prior to administration of araAMP. Brains were assayed for ^{3}H content at the indicated times after araAMP administration by the combustion-scintillation counting method. Data are presented as mean ± S.D., n = 5 for all points.

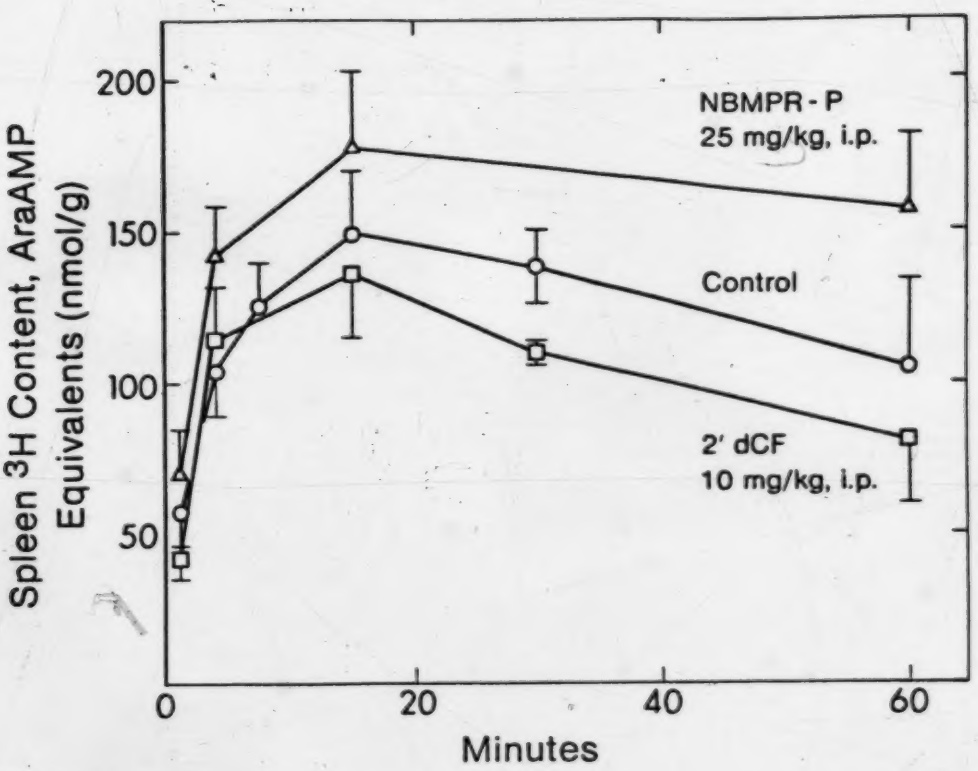


Fig. 9. Spleen count of araAMP metabolites. All animals received [2,8-^{3}H]araAMP (15 mg/kg, i.v.). Mice in the pretreated groups received 2'-dCF (10 mg/kg, i.p.) or NBMPR-P (25 mg/kg, i.p.) 30 min prior to administration of araAMP. Spleens were assayed for ^{3}H content by the combustion-scintillation counting method at the indicated times after araAMP administration. Data are presented as mean ± S.D., n = 5 for all points.

NBMPR-P, which one might expect to reduce rates of araA uptake into the organ, resulted in a substantial enhancement of accumulation. Other data will be presented in a later chapter which suggest that araA is transported and metabolized in spleen in a manner unlike the conventional view of the handling of nucleosides by tissues.

e. Kidney

The post administration decline in the araAMP-derived isotope content of kidney was much more rapid (see Figure 10) than in the tissues mentioned previously. Presumably, this decline resulted from a very rapid elimination of araA metabolites into urine. Prior treatment with NBMPR-P, which was shown above to slow urinary excretion of drug-derived isotope, also significantly reduced the isotope content of kidney 1 min after administration of araAMP. 2'-dCF treatment had a similar effect on the time course of kidney content of araAMP and metabolites, but also resulted in a significantly lower concentration in kidney 4 min after drug administration than that in the control animal.

The protracted elevation of kidney isotope content (47.2 ± 2.8 nmol/g, 4 hr after araAMP administration) relative to plasma levels seen following the rapid initial decline would suggest, because of an apparent independence from plasma concentration, that at late time points, major kidney metabolites were retained intracellularly, not in tubular fluid. Alternatively, this may have represented active secretion in the kidney.

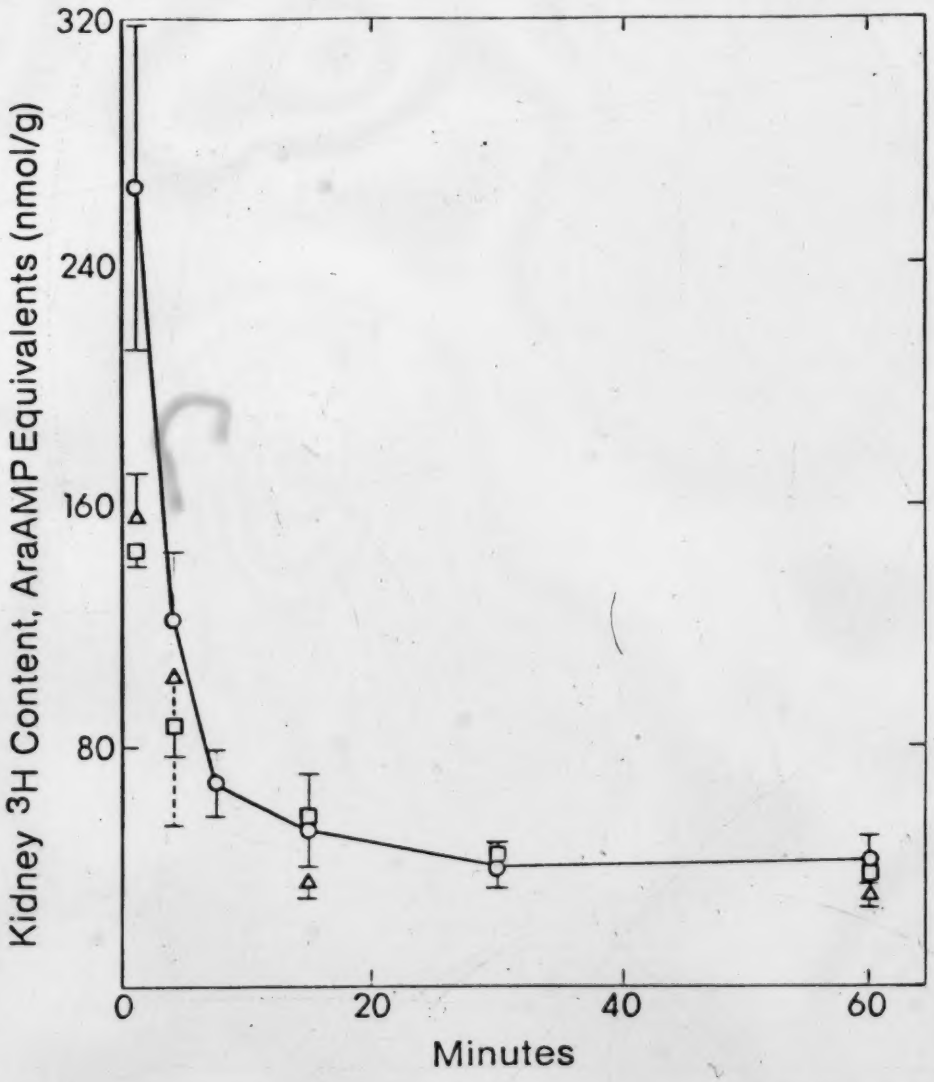


Fig. 10. AraAMP-derived ^{3}H in the kidney. All animals received 2,8-^{3}H araAMP (15 mg/kg, i.v.). Mice received 2'-dCF (10 mg/kg, i.p.; □), NBMPR-P (25 mg/kg, i.p.; △), or no pretreatment (○) 30 min prior to the administration of araAMP. Kidneys were assayed for ^{3}H content by the combustion-scintillation counting method at the indicated times after araAMP administration. Data are presented as mean ± S.D., n = 5 for all points.

B. Discussion

1. Concentration of AraAMP and Metabolites

All tissues assayed with the exception of brain contained higher concentrations of araAMP-derived nonvolatile ^{3}H than did plasma. In most tissues this concentrative effect probably resulted from intracellular metabolism of permeant araA. AraA would be effectively removed from transport equilibria (Plunkett and Cohen, 1977) if, after entering the cell, it was phosphorylated to araAMP, araADP and araATP. Preliminary data on metabolites of araAMP in liver (not shown) suggest that this metabolic trapping explains the accumulation of metabolites of araAMP observed in that tissue: nucleoside concentrations in liver appeared to be in equilibrium with plasma. Substantial concentration of ^{3}H resulted from the presence of araA phosphates.

2. Distribution in Spleen

The observation that araH and araA were the principal metabolites of araAMP found in the spleen (data presented later in this thesis) excluded trapping by phosphorylation as the basis of the concentration of araAMP metabolites in that tissue. Peak concentrations of araAMP-derived ^{3}H were observed in spleen only after levels of ^{3}H in plasma had fallen considerably from their peak. ^{3}H Concentrations in spleen were 4-fold higher than in plasma (126:31) 7.5 min after drug administration, yet concentrations in the spleen continued to rise after this point, an obvious concentrative effect.

Pretreatment with NBMPR-P resulted in ^{3}H concentrations in spleen which were elevated in comparison to control. A possible explanation for these observations would be to postulate the existence of an active, concentrative, NBMPR-insensitive nucleoside transporter, however, no precedents are known for such a mechanism.

3. Distribution to Kidney

The ^{3}H content of kidney followed a very different time course from that of spleen. Following i.v. administration of labelled araA or araAMP, the ^{3}H content of kidney had reached a peak before 60 sec after injection. Prior treatment with NBMPR-P or 2'-dCF did not affect this result. Following a rapid, apparently exponential decline in kidney ^{3}H content from 1-15 min, kidney ^{3}H concentrations did not change significantly from 15 min to 4 hr after administration of labelled araAMP. This time course could reflect two components of kidney metabolite content. AraAMP metabolites undergoing urinary excretion would readily explain the exponential phase, while intracellular metabolic trapping of araA metabolites might account for the stable phase.

Kinkel and Buchanan (1975) have described a protracted period of relatively constant urinary excretion of araH in the presence of greatly diminishing plasma concentrations of araA metabolites in man. They conclude that the excreted drug is derived from some compartment other than plasma. A prolonged constant rate of urinary excretion, independent of plasma arabinoside concentrations, might

also explain the prolonged elevation of araAMP metabolite concentrations in the kidney observed in this study. This suggests the possibility that there is active secretion of arabinosides in the kidney.

A number of reports (Marker et al., 1978; Sacks et al., 1979; Tyrrell et al., 1980; Friedman, 1981) describe enhanced toxicity of araA or araAMP in patients with impaired renal function. These observations emphasize the importance of the kidney in the elimination of both drugs.

The concentration of araAMP equivalents found in kidney 60 sec after drug administration was almost 100 nmol/g higher than the highest value in any other tissue. Pretreatment with either NBMPR-P or 2'-dCF had little effect on the time course of ^{3}H in kidney, except to significantly reduce the ^{3}H concentration observed 60 sec after administration. Prior treatment with either drug also slowed the appearance of araH in plasma (data presented subsequently), suggesting that the reduced renal ^{3}H content at early time points may have reflected a difference between the urinary excretion of araA and araH.

The kidney content of arabinoside metabolites was lower after i.v. administration of araA than after that of araAMP at the three time points assayed. This may have reflected higher phosphomonoesterase activity in kidney than in other tissues of the mouse (LePage et al., 1972): one would expect that araA produced by dephosphorylation of araAMP in the kidney would first become accessible to transport in that tissue.

4. Distribution in Liver

Concentrations of araAMP-derived ^{3}H in liver following i.p. administration were not elevated relative to those found after i.v. administration. A substantial portion of the araAMP molecules in an i.p. injected dose would be expected to enter the hepatic portal circulation before entering the systemic circulation (Sartorelli and Upchurch, 1962). Since this "first pass" effect did not result in enhanced concentrations of araAMP metabolites in liver, relative to those derived from i.v. injection, competition with other tissues for drug in systemic circulation does not appear to be a limiting factor in liver accumulation of araAMP-derived ^{3}H. Similarly, no difference was found between drug-equivalent contents derived from araA and araAMP, administered either i.v. or i.p. Thus, dephosphorylation did not appear to be the limiting step in liver accumulation.

Prior treatment with 2'-dCF dramatically enhanced the araAMP-derived ^{3}H content of liver at all time points beyond 1 min after administration of araAMP. This suggests that deamination to araH competed with phosphorylation for araA. AraH is a likely substrate for the nucleoside transporter. Since araH does not appear to undergo appreciable phosphorylation (Brink and LePage, 1964), it would not be metabolically trapped and, accordingly, efflux from hepatocytes would be mediated and would proceed along concentration gradients. Competition between these two pathways would seem to be a primary factor in limiting liver retention of araA metabolites.

AraAMP-derived ^{3}H concentrations were reduced in liver when araAMP administration was preceded by treatment with NBMPR-P. This suggested that a significant fraction of araAMP equivalents entered hepatic cells by way of an NBMPR-P-inhibitable nucleoside transporter. Four minutes after administration of araAMP, however, the liver ^{3}H content in NBMPR-P-treated mice remained at over 80% of the value found in untreated animals. This may have reflected (a) a dosage of NBMPR-P insufficient to substantially inhibit nucleoside transport in the liver, or (b) entry of araAMP (or its metabolites) into hepatic cells by a mechanism insensitive to NBMPR.

In man, liver disease does not appear to affect plasma half-lives of araAMP, araA or araH, nor is accumulation of araAMP metabolites seen in the plasma of patients with hepatic dysfunction (Whitley et al., 1980; Preksaitis et al., 1981). Despite the lack of effect of compromised hepatic function on these pharmacokinetic parameters, Preksaitis and coworkers observed neurological toxicity only in patients with liver disease. Myelosuppression, though not severe, was enhanced in patients with compromised hepatic function, though this may have been a result of the disease state, not treatment.

The liver is obviously an important organ in the detoxification of araAMP, although the serum pharmacokinetic data do not reflect that fact. This role in the metabolism of araAMP was reflected in mice by the observation that 1 hr after drug administration, 6% of the administered araAMP equivalents were retained in the liver. By this point, when roughly 70% of the administered dose had been lost by urinary excretion or oxidative degradation, the total isotope

content of liver exceeded that of other tissues, despite higher concentrations observed in the spleen.

5. Distribution in Brain

The lowest concentrations of araAMP-derived ^{3}H found in any tissue were in the brain. Pretreatment with 2'-dCF increased the ^{3}H concentration found in brain, but not as dramatically as in the case of liver. Prior treatment with NBMPR-P resulted in a reduction of 50% in brain content of araAMP equivalents at all three time points evaluated. Both agents could have utility in altering the therapeutic index of araAMP.

Exclusion of araAMP metabolites from the brain by NBMPR-P pretreatment suggests a potential therapeutic application for the latter. In treatment of chronic recurrent hepatitis, neurotoxicity appears to be a limiting factor in the antiviral use of araAMP (Whitley et al., 1980). Pretreatment with NBMPR-P might reduce this toxicity by reducing araAMP access to the CNS. Although liver accumulation of araAMP-derived isotope was also reduced following treatment with NBMPR-P, the latter's effect in that tissue was not nearly as pronounced as in brain. Fifteen minutes after administration of araAMP, the ratio of drug-derived isotope concentrations in liver and brain was 15:1 in unpretreated animals, whereas prior treatment with NBMPR-P raised this ratio to 37:1.

Although prior treatment with 2'-dCF increased brain accumulation of araAMP metabolites, this combination might also be useful in

alleviating neurological toxicity in hepatitis patients for the following reason. Fifteen minutes after administration of araAMP, the ratio of liver/brain ^{3}H contents was 25:1 in 2'-dCF pretreated mice. This value was higher than in the control, reflecting a larger proportional increase in liver concentrations than in brain. If the total administered dose could be lowered, while maintaining efficacious antiviral levels in the liver, entry into CNS might also be reduced, with a concomitant reduction in toxicity.

The recent evidence of Friedman (1981) which suggests that the active neurotoxic agent may be araH also supports the possibility that 2'-dCF might reduce the neurotoxicity of araAMP. Inhibition of deamination would prevent formation of the putative neurotoxic metabolite, in addition to the alteration in tissue distribution produced by the drug. However, the greatly enhanced cytotoxicity observed with araA in the presence of 2'-dCF (Cass et al., 1980) must also be an important consideration in this approach.

The low concentrations of araAMP metabolites observed in brain relative to other tissues are not consistent with the observation (Glazko et al., 1975) that "cerebrospinal fluid levels (of araA-derived isotope) in man are somewhat lower than plasma levels," but "indicate ready passage of the drug or its metabolites into the central nervous system." This inconsistency does not appear to stem from species differences. The plasma and cerebrospinal fluid levels described by Glazko and coworkers were assayed 4-7 hr after araA administration. The present study indicated that after such post-

administration intervals in mice, brain concentrations are indeed higher than those in plasma, but much higher values are found in kidney (21-fold higher than brain concentrations), liver (20-fold), and spleen (18-fold). Certainly the data from this study do not "indicate ready passage of the drug or its metabolites into the central nervous system" when accumulation is compared to that in other tissues.

The apparently limited access of araAMP to the brain (and araA does not appear to differ significantly in this regard) appears inconsistent with the drug's pronounced antiviral efficacy in the treatment of human herpetic encephalitis. The possible effects of virus-induced lesions on the permeability of the blood brain barrier to araAMP and its metabolites are not known, however. Although herpetic encephalitis does not result in the widespread meningeal inflammation and disruption characteristic of the bacterial meningitides, localized permeability alterations at infected foci might enhance therapeutic availability of the drug at the site of pathology.

6. Plasma and Blood Time Courses of AraAMP-Derived ^{3}H

Nonvolatile ^{3}H in plasma 15 min after i.v. administration of [2,8-^{3}H]araAMP was entirely in the form of araH (as is the case in man) and, therefore, the half-life of the isotope at this and longer time points can be regarded as an elimination half-life for araH. The 19.8 min half-life for this metabolite in mice is much shorter

than the 3.5 ± 1.2 hr reported in man (Preksaitis et al., in press). The longer half-life in man might be anticipated, since in man 52.2 ± 10.2 percent of the administered dose of araAMP is excreted in 24 hr, principally as araH. This value is similar to the one hour urinary excretion in mice of 46.8 ± 3.1 percent of the administered dose. Thus, the more rapid urinary excretion of araH in mice relative to that in humans is reflected in a shorter plasma half-life of araH.

Pretreatment with NBMPR-P might be expected to elevate plasma levels of araAMP-derived ^3H as a result of blocked entry into tissues. Instead, plasma concentrations appeared to decline more rapidly in NBMPR-P treated mice than in untreated mice. Enhanced urinary excretion of araAMP metabolites did not account for this result; in fact urinary excretion of araAMP-derived isotope was reduced by NBMPR-P pretreatment. The cause of this NBMPR-P-enhanced plasma clearance of nonvolatile ^3H evidently resides in an enhanced rate of catabolism of araAMP, with an increased loss of ^3H as ^3H_2O as a result (see the following chapter). This catabolism may occur in a tissue in which uptake of araAMP metabolites is not inhibited by pretreatment with the transport inhibitor.

Treatment of mice with 2'-dCF did not substantially change the time course of total araAMP radiometabolites in plasma, despite the fact that this treatment dramatically altered the relative amounts of those metabolites (data presented subsequently).

Although mouse erythrocytes *in vivo* concentrated araAMP-derived isotope from plasma, the concentrations achieved are not as high as in other mouse tissues, nor as high as concentrations of metabolites of another adenosine analogue, tubercidin, in mouse erythrocytes (Kolassa *et al.*, in press). ^{3}H trapped intracellularly as ^{3}H-tubercidin phosphates was 42-fold more concentrated in whole blood than in plasma 2 hr after a dose of 15 mg/kg tubercidin i.v. At 1 and 4 hr after i.v. administration of 15 mg/kg of araAMP, whole blood/plasma isotope ratios of 1.7 and 3.4, respectively, were observed in this study. This difference probably arises from two differences in the metabolism of these two adenosine analogues. AraA is phosphorylated in cells by deoxyadenosine kinase and deoxycytidine kinase (Brockman *et al.*, 1980; Krenitsky *et al.*, 1976), but is a very poor substrate for mammalian adenosine kinase; in contrast, tubercidin is an even better substrate for adenosine kinase than adenosine (Schnebli *et al.*, 1967). Since levels of the two kinases important in the phosphorylation of araA would be expected to be low or negligible in mature erythrocytes (in which polynucleotide synthesis does not occur), araA trapping by phosphorylation should be much less prominent than trapping of tubercidin. Additionally, since tubercidin is not a substrate for adenosine deaminase, deamination is not an alternate pathway for that drug.

7. Effects of Administrative Protocol on Tissue Distribution of AraAMP

The effects of altering the route and regimen of araAMP administration were assessed in the hope of recognizing useful therapeutic strategies. Admittedly, it is difficult to extrapolate from tissue distribution of araAMP-derived ^{3}H in the mouse to antiviral activity in specific tissues of interest in man; hopefully, these data may be of some value in influencing the access of araA to human tissues.

Following either of the i.v. infusion regimens, araAMP concentrations in all tissues did not exceed those attained at peak tissue concentrations (or 1 hr levels) following i.v. injection of araAMP. AraAMP equivalent concentrations in brain, liver and kidney 4 hr after i.v. injection remained as high as, or higher than the concentrations (probably near peak levels) found in those tissues 15 min after termination of infusion. A therapeutic advantage for i.v. infusion (which might result from an increase in the area under a concentration versus time plot for any of these tissues) seems unlikely in light of these data.

Significantly higher ^{3}H contents were seen in uterus 60 min after i.p. than i.v. injection of araAMP. This might indicate a topical application effect, since solution injected into the peritoneal cavity would be expected to come into contact with the uterus. This interpretation is not supported by ^{3}H levels in other tissues where direct contact would occur following i.p. injection, because ^{3}H concentrations in those tissues following i.p. injec-

tion of araAMP were lower than, or unchanged from those levels found after i.v. injection.

If ^{3}H distribution of araAMP in mice is a reasonable model for the access of araAMP to tissues in humans, beneficial results in terms of tissue distribution and practical ease of administration would result from the fastest feasible administrative regimen. Presumably this would imply i.v. bolus injection or rapid i.v. infusion.

IV. Recovery of Administered ^3H

A. Results

1. Formation of ^3H_2O from [2,8-^3H]araA and [2,8-^3H]araAMP

Initial attempts to account for the total ^3H administered as araAMP were unsuccessful because a substantial portion of ^3H administered appeared to be released in vivo as ^3H_2O. Table 5 lists the fraction of the plasma ^3H content which was in a nonvolatile form 1 hr after administration of araA (11 mg/kg, i.v.) or araAMP (15 mg/kg, i.v.). Also presented are the ^3H components found in nonvolatile form when administration of araAMP was preceded by treatment with NBMPR-P, 2'-dCF or allopurinol.

In animals which received only araAMP, 60% of the ^3H content of plasma 60 min after administration of araAMP was in the form of ^3H_2O. Since the original ^3H-labelled compounds were stable (i.e. the tritium label did not exchange with water) for periods in excess of 3 months in sterile 0.15 M NaCl at room temperature, this labilization appeared to be metabolic rather than physicochemical. The nonvolatile fraction of plasma ^3H found 60 min after administration of labelled araA (34%) was similar to that found after araAMP (40%).

Prior treatment with NBMPR-P increased to 83% the proportion of plasma isotope found as ^3H_2O after administration of ^3H-araAMP. This finding suggested that the extent of labilization varied

Table 5. Nonvolatile ^{3}H in plasma 60 min after administration of [2,8-^{3}H]araAMP or [2,8-^{3}H]araA. Mice recived araAMP (15 mg/kg, i.v.) or araA (11 mg/kg, i.v.). Pretreated groups received NBMPR-P (25 mg/kg, i.p., 30 min before araAMP), 2'-dCF (10 mg/kg, i.p., 30 min before araAMP) or allopurinol (10 mg/kg, i.p. as a suspension, 60 min before araAMP). Plasma samples from each animal were mixed with scintillation fluid immediately after sampling or dried in a freeze drier, redissolved and mixed with scintillation fluid. The ^{3}H content of dried plasma samples was expressed as a percentage of the total ^{3}H content of the same plasma samples (assayed without drying).

Labelled drug administered	Pretreatment	Percentage of ^{3}H in plasma in nonvolatile form (Mean ± S.D.)[+]
araAMP	none	39.6 ± 4.2 %
araA	none	34.2 ± 2.0 %
araAMP	NBMPR-P	16.6 ± 0.9 %
araAMP	2'-dCF	100.9 ± 4.3 %
araAMP	allopurinol	101.7 ± 3.2 %

[+] n = 5 for all groups.

in different tissues, since drug accumulation in various tissues was altered following treatment with the transport inhibitor.

Treatment with either 2'-dCF or allopurinol totally inhibited labilization, suggesting that at least two metabolic steps, deamination and oxidative catabolism, were required to remove ^{3}H from the purine ring of araAMP.

2. Recovery of Administered Radioactivity

The fraction of administered radioactivity which could be accounted for 60 min after administration of [2,8-^{3}H]araAMP was determined by three methods. The results are presented in Table 6.

By adding a measured mean value for 1 hr urinary excretion of label to the total radioactivity representing the summed carcass and tissue ^{3}H contents (determined by the combustion-scintillation counting procedure), it was possible to account for only 83% of the administered ^{3}H (balance sheet method). Prior administration of NBMPR-P reduced the recovery determined in this manner to less than 60%. Though based on approximations of skeletal muscle mass, blood volume, and urinary excretion, both these values for recovery correspond closely to the values determined by combustion and scintillation counting of whole body homogenates.

Total body $^{3}H_2O$ content was calculated by multiplying estimated total body H_2O (Green, 1966) by the volatile ^{3}H concentration of plasma samples, since $^{3}H_2O$ would be expected to be in equilibrium with all body fluids. When this value for total

Table 6. Recovery of ^3H administered as [^3H]araAMP. Mice received [2,8-^3H]araAMP (15 mg/kg, i.v.) 60 min before sampling. Pretreated mice received NBMPR-P (25 mg/kg) 30 min before administration of araAMP. Balance sheet recovery was determined by summing ^3H contents of sampled tissues, as determined by combustion and scintillation counting. Samples of whole body homogenates were spotted onto chromatography paper, dried and assayed for ^3H content by combustion and scintillation counting. A calculated value for ^3H_2O in body fluid (see previous section) was added to the balance sheet recovery for each animal to yield the volatilization corrected recovery. All values are expressed as the fraction of administered ^3H which could be accounted for by the method indicated.

Pretreatment	Balance sheet recovery (mean ± S.D.)[+]	Whole body homogenate recovery (mean)[*]	Balance sheet recovery, volatile ^3H corrected (mean ± S.D.)[+]
none	83.0 ± 6.9 %	81 %	101.3 ± 9.1 %
NBMPR-P	58.5 ± 6.3 %	61 %	95.2 ± 13.2 %

[+] n = 5

[*] n = 2

$^{3}H_2O$ was added to the recovery estimate made by the balance sheet method, a value for recovery corrected for volatile ^{3}H was derived for each animal. Thus, it appeared that all the label administered could be recovered by combustion of tissues, except the ^{3}H lost in drying after metabolic conversion to $^{3}H_2O$.

B. Discussion

The inability to account for 17-42% (with or without pretreatment, Table 6) of the ^3H administered as [2,8-^3H]araAMP, 60 min after drug administration, made interpretation of data from tissue distribution studies difficult. This loss of label was explained by the observation that ^3H not accounted for by balance sheet recovery had been lost as ^3H_2O during drying of tissues. Thus it became important to establish the mechanism by which ^3H_2O was formed from [2,8-^3H]araAMP.

Pretreatment with 2'-dCF totally inhibited the appearance of ^3H_2O, suggesting that ^3H in the 2 or 8 position of the purine ring in araH may have been labile. This possibility was made unlikely by the observation that treatment of mice with allopurinol inhibited the production of ^3H_2O completely, without affecting deamination.

The stability of ^3H-araH was verified as follows. A mouse was injected with [2,8-^3H]araAMP (15 mg/kg, i.v.) 60 min after treatment with allopurinol (10 mg/kg, i.p. as a suspension), a potent inhibitor of xanthine oxidase (Elion, 1978). The urine was collected and araH was isolated by TLC. This ^3H-araH after being sterilized by filtration to prevent bacterial breakdown, was stable at room temperature (i.e. exchange of ^3H with H_2O was not detectable).

Pretreatment with allopurinol abolished the formation of ^3H_2O from araAMP in the mouse. This evidence implicated xan-

thine oxidase as the enzyme responsible for production of ^3H_2O, apparently arising from the oxidative catabolism of araAMP metabolites. A similar inhibition by allopurinol of labilization from ^3H-araA has been reported in dogs (Glazko et al., 1975), and recent evidence suggests that oxidative catabolism may be a significant route for detoxification of araA in man (Friedman, 1981).

Although studies on the substrate specificity of xanthine oxidase have not included araA, purines with large substituent groups at the 9 position are generally poor substrates for xanthine oxidase (Krenitsky, 1972). In support of this view, we found that bovine milk xanthine oxidase (Sigma Chemical Co., St. Louis, Missouri, Lot 58B-2380-1) did not release ^3H_2O from [2,8-^{3}H]araH under conditions which permitted oxidation of hypoxanthine (Krenitsky et al., 1972). We were also able to detect oxidation of hypoxanthine under those conditions. This suggests that cleavage of the N-glycosidic linkage with release of hypoxanthine took place before oxidative catabolism occurred. Since 2'-dCF inhibited formation of ^3H_2O, cleavage of araA does not appear to be the initial step in formation of ^3H_2O.

LePage and Brink (1964b) were unable to detect cleavage of araA and araH by extracts of a number of mouse tissues which cleaved adenosine. These authors did, however, observe cleavage of araA and araH by murine tumor lines TA3 and L1210, suggesting that some non-malignant mouse tissue might share this activity. The enzyme(s) responsible for this cleavage is obscure, since neither araA nor

araH is a substrate for purine nucleoside phosphorylase (R.P. Agarwal, personal communication; Parks et al., 1981) or 5'-methylthioadenosine phosphorylase (Parks et al., 1981). While araA is cleaved to adenine and an unidentified sugar moiety by S-adenosylhomocysteine hydrolase (Abeles et al., 1980), the adenine so produced is enzyme-bound, and thus would not be subject to catabolism.

It is postulated that the catabolism of araA proceeds as follows. AraA is deaminated to araH which is subsequently cleaved with release of hypoxanthine. The hypoxanthine so generated is oxidized to xanthine and then to uric acid by xanthine oxidase, with release of ^{3}H from the 2 and 8 positions of the purine ring as $^{3}H_2O$. The resulting unlabelled urate is excreted after cleavage to allantoin.

Since $^{3}H_2O$ evidently arises from oxidation of hypoxanthine, volatile ^{3}H lost from tissue distribution studies stems from an antivirally inactive metabolite. This loss of label would have no therapeutic significance.

Pretreatment with NBMPR-P resulted in an increase in the amount of ^{3}H lost from ^{3}H-araAMP in the form of $^{3}H_2O$. This suggests that oxidative catabolism occurs primarily in a tissue insensitive to NBMPR-P inhibition of uptake. The NBMPR-P-enhancement of oxidative degradation of ^{3}H-araA appears to explain the earlier observation (Figure 7) of an enhanced rate of decline of nonvolatile ^{3}H in plasma with no concomitant increase in tissue concentrations of ^{3}H.

V. Metabolism of AraA and AraAMP

Results

1. Plasma Metabolites Following Treatment With AraA and AraAMP

Concentrations of radiometabolites in plasma were determined at 3 intervals after the i.v. administration of [2,8-^{3}H]araAMP to mice. Also examined were the effects of prior treatment with 2'-dCF and NBMPR-P on formation of these metabolites. Neutralized PCA extracts of plasma were analyzed by TLC, and radiometabolite concentrations were determined by scintillation counting of ^{3}H which accompanied authentic arabinoside markers. The results are shown in Table 7.

In the plasma of animals that had not received pretreatment, the principal metabolite of araAMP was araH; >90% of the ^{3}H applied to TLC plates could be accounted for as araA, araH, and araA nucleotides. As early as 4 min after i.v. administration of ^{3}H-araAMP, araH was the principal metabolite seen in plasma, and after 15 min it was the only metabolite which remained in detectable quantities.

Prior treatment with 2'-dCF (10 mg/kg, i.p., 30 min before araAMP) resulted in almost total inhibition of deamination. AraA was the principal metabolite at all three time points. Treatment with 2'-dCF had little effect on the rate of dephosphorylation of araAMP.

The administration of NBMPR-P, 30 min prior to that of ^{3}H-araAMP, hastened the decline in plasma ^{3}H concentration. AraA concentrations 1 and 4 min after administration were higher in the

Table 7. Metabolites of araAMP in plasma. Neutralized PCA extracts of plasma samples taken at the specified intervals after administration of [2,8-^{3}H]araAMP (15 mg/kg, i.v.) were analyzed by TLC and scintillation counting. Pretreated mice received NBMPR-P (25 mg/kg, i.p.) or 2'-dCF (10 mg/kg, i.p.) 30 min prior to administration of araAMP.

Pretreatment	Interval to sampling (min)	Plasma concentrations (μM)+					
		AraAMP		AraA		AraH	
None	1	21.9	23.7	26.5	29.1	16.0	17.8
NBMPR-P	1	14.5	18.5	58.5	70.1	7.0	7.6
2'-dCF	1	22.2	24.3	43.6	46.4	5.0	6.1
None	4	5.8	6.6	4.0	5.4	28.9	33.3
NBMPR-P	4	2.7	3.5	7.4	11.6	16.2	18.6
2'-dCF	4	5.0	5.4	26.1	28.5	3.1	2.7
None	15	0	0	0	0	22.0	27.8
NBMPR-P	15	0	0	<1	<1	12.3	14.5
2'-dCF	15	0	0	11.0	15.8	2.0	2.6

+ Data are presented for two replications at each point.

NBMPR-P pretreated animal than in the control. This elevation may also have arisen from a transient inhibition of deamination, as araH levels were lower than control at both time points. By 15 min after araAMP administration, however, araH was the principal metabolite in the plasma of NBMPR-P treated animals, with araA concentrations having fallen below 1 μM.

The plasma metabolite concentrations following i.v. administration of araAMP were compared with those of araA administered at equimolar dosage by the same route. The results are presented in Table 8. AraH levels following administration of araAMP appeared lower than those resulting from araA administration, reflecting the resistance to deamination of araAMP. AraA concentrations appeared lower following administration of the nucleotide. A substantial portion the injected araAMP in plasma (6.7 μM) remained in that form 4 min after administration. By 15 min (see Table 7), neither araAMP nor araA could be detected in the plasma of animals receiving araAMP.

2. Spleen Metabolites of AraAMP

The radiometabolites of araAMP in spleen were assayed 15 and 60 min after the i.v. administration of [2,8-^{3}H]araAMP. The effects of pretreatment with NBMPR-P and with 2'-dCF were also assessed. The results are presented in Table 9. Remarkable in all three groups was the large component of drug-derived isotope in the form of unphosphorylated nucleosides. The ratio of splenic arabinoside content to that of plasma 15 min after administration of araAMP was

Table 8. Plasma metabolites following araA and araAMP administration. Neutralized PCA extracts of plasma samples taken at the specified intervals after administration of either araAMP (15 mg/kg, i.v.), or araA (11 mg/kg, i.v.) were characterized by scintillation counting of isolated metabolites on TLC plates.

Pretreatment	Interval to sampling (min)	Plasma concentrations (μM)+					
		AraAMP		AraA		AraH	
araAMP	1	21.9	23.7	26.5	29.1	16.0	17.8
araA	1	-	-	38.7	43.3	19.9	22.1
araAMP	4	5.8	6.6	4.0	5.4	28.9	33.3
araA	4	-	-	32.8	39.8	5.0	5.6

+ Data are presented for two replications at each point.

Table 9. AraAMP metabolites in the spleen. Neutralized PCA extracts of spleens which had been frozen by a Wollenberger clamp at the specified interval following injection of [2,8-^{3}H]araAMP were analyzed for radiometabolites by TLC and scintillation counting. Pretreated animals received NBMPR-P (25 mg/kg, i.p.) or 2'-dCF (10 mg/kg, i.p.) 30 min before administration of araAMP. The metabolites listed accounted for >90% of the ^{3}H content of spleen extracts.

Pretreatment	Sampling time	Spleen metabolite content (μM)+									
		AraATP		AraADP		AraAMP		AraA		AraH	
None	15 min	9.1	11.3	3.1	2.7	2.9	2.3	2.0	2.6	111.2	123.8
NBMPR-P		6.5	10.7	2.0	2.8	2.0	2.2	<1	<1	150.6	182.2
2'-dCF		12.7	14.1	3.0	3.4	1.3	1.7	106.1	116.9	2.8	4.6
None	1 hr	20.6	30.2	5.9	8.1	4.0	4.0	<1	<1	56.1	71.5
NBMPR-P		17.9	18.9	5.4	6.8	7.6	8.0	<1	<1	107.6	135.9
2'-dCF		16.4	20.0	4.1	4.9	1.0	1.6	44.3	53.9	3.9	4.5

+ Data are presented for two replications at each point.

12.4 in NBMPR-P treated mice, 7.3 in those treated with 2'-dCF, and 4.8 in animals which were not pretreated. Unphosphorylated arabinonucleosides were therefore retained within spleen cells against a concentration gradient in all three cases.

In animals not receiving pretreatment, the principal metabolite of araAMP found in spleen was araH. The fraction of nonvolatile ^{3}H in the form of araH exceeded the sum of all other metabolites at both time points. AraA mono-, di- and triphosphates were found in the spleen 15 min after drug administration, and the concentration of each was higher 60 min after administration. The low concentrations of araA assayed 15 min after administration fell to barely detectable levels by 60 min.

Pretreatment with NBMPR-P resulted in concentrations of araH in the spleen that were markedly higher than the control at both time points. As in control mice, araA nucleotide levels in spleen rose between the 15 and 60 min time points. AraA was barely detectable at both time points. The principal metabolite of araAMP in spleen at both time points following 2'-dCF pretreatment was araA. As with plasma, the metabolite content of spleen suggested an almost total inhibition of deamination of araAMP and metabolites following 2'-dCF treatment. Concentrations of arabinonucleotides resembled those seen in the control and NBMPR-P pretreated groups.

In control and both pretreated groups of mice, total nucleotide concentrations were higher at the second time point, while total nucleoside contents were reduced.

B. Discussion

1. Plasma Metabolites

Sixty seconds after administration of ^{3}H-araAMP, 66% of the ^{3}H in plasma was found in the form of araA and araH. This rapid dephosphorylation shows that, in mice, araAMP is not a long lasting prodrug. By 15 min after administration, araH was the only metabolite detectable in the plasma of animals receiving araAMP without pretreatment.

LePage et al., (1972) have described the rapid catabolism of araAMP in mice, but have argued that differences between humans and mice in respect to renal phosphomonoesterase activity (0.6–2.4 catalytic units/g in human kidney, 238 units/g in mouse kidney) prevented extrapolation from the murine to the human system. These investigators also described sustained concentrations of araA and araAMP in the plasma of patients treated with araAMP.

Recent studies (Tyrrell et al., 1980; Preksaitis et al., 1981) have been unable to reproduce such sustained levels of undeaminated metabolites in patients. Furthermore, these workers and others (Whitley et al., 1980) demonstrated the presence of araH as a major plasma metabolite of araAMP, in contrast to LePage et al., who were unable to detect that nucleoside after i.v. administration of araAMP.

A comparison of the data presented in this chapter with the data of Preksaitis et al. (1981) suggests that in humans and mice, araAMP is metabolized in a very similar manner. In both species, araAMP

and araA were undetectable in plasma 15 min after the i.v. administration of araAMP.

A comparison of the metabolites of araA and araAMP in plasma 1–4 min after drug administration suggested that formation of araH was slower following administration of araAMP. This resistance did not result in sustained plasma levels of araA, however. AraAMP from plasma is unlikely to enter cells in significant quantities without prior dephosphorylation, so araAMP *per se* in plasma is probably antivirally inactive (Plunkett and Cohen, 1976). At early time points, concentrations of araA are lower following administration of araAMP than of the parent nucleoside, therefore no therapeutic advantage seems likely to result from the deaminase resistance of araAMP.

The plasma metabolite data presented here, in combination with the tissue distribution studies described earlier, support the present view (Tyrrell *et al.*, 1980) that araAMP should be regarded simply as a soluble prodrug of araA. This conclusion is also supported by the very similar results for the two drugs observed in comparative toxicology studies (Kurtz *et al.*, 1977).

The few reports which attributed lower antiviral potency to araAMP relative to the parent nucleoside (e.g. Sidwell *et al.*, 1973) compared mass equivalent, rather than molar equivalent dosages of the two drugs. In other animal studies (Sidwell *et al.*, 1973; Sloan, 1975), topically or parenterally administered araAMP was

shown to be a more potent antiviral agent (often because of its solubility), even when compared to gram equivalent doses of araA. This suggested that araAMP exhibited at least equal antiviral activity to araA, with equivalent or less toxicity. These findings, as well as the results presented in this thesis, support the use of araAMP as a simple prodrug form of araA.

Pretreatment with 2'-dCF greatly prolongs the half-life of araA in plasma. As araA is probably the principle active antiviral metabolite in plasma, the combination of araAMP and 2'-dCF offers promise in enhancing antiviral potency. Careful dosage adjustment may be necessary to improve the therapeutic index of treatment, however, as the combination of araA (or araAMP) and 2'-dCF is much more cytotoxic and immunosuppressive than either agent administered independently (Plunkett and Cohen, 1979; Shannon et al., 1980; Lum et al., 1980).

NBMPR-P appears to transiently inhibit deamination of araA in plasma. This effect might result if the transport inhibitor slows the uptake of araAMP by a tissue, such as liver, which has high levels of adenosine deaminase activity (Ho et al., 1980).

2. Metabolism in Spleen

The view is generally held that accumulation of nucleoside drugs in cells and tissues against an apparent concentration gradient is a result of intracellular metabolic trapping (Cohen, 1975; Plunkett and Cohen, 1977; Carson et al., 1979). Nucleosides enter the cell

by facilitated diffusion (Paterson et al., 1981), and are phosphorylated to nucleotides. Because the plasma membrane is not permeable to nucleotides they accumulate within the cell (Plunkett and Cohen, 1977). Preliminary experiments (not shown) indicated that the araH-araA content of liver appeared to be in equilibrium with plasma, while hepatic concentration of ^{3}H above plasma levels probably resulted from intracellular trapping of arabinonucleotides.

The metabolite concentrations in spleen indicate that intracellular phosphorylation was not the mechanism resulting in concentration of araA metabolites in that tissue. In animals receiving NBMPR-P or no pretreatment, the principal metabolite observed in spleen was araH. Pretreatment with 2'-dCF resulted in high concentrations of araA. The ATP/ADP ratio measured by HPLC for one of the extracts was 3.1:1. This value corresponds well to those in the literature for a number of tissues (Keppler et al., 1970; Schultz and Lowenstein, 1978), and argues that these concentrations of arabinonucleosides did not arise from the breakdown of nucleotides due to an inadequate extraction procedure. The high concentrations of araH demonstrated in spleen by TLC methods were confirmed by HPLC analysis (A.F. Almeida, K.G. Pippus and E.S. Jacobs, unpublished results).

Spleens in NBMPR-P treated mice accumulated more araAMP-derived ^{3}H than did those in unpretreated mice. Spleen accumulation of metabolites was highly concentrative, yet in all cases, arabinotides

accounted for less than 40% of the ^{3}H retained. Both these observations could be explained by the existence of an active, concentrative, NBMPR insensitive nucleoside transport mechanism in the spleen. More work is required to evaluate this possibility. It is interesting to note that splenic accumulation of tubercidin administered i.v., is also enhanced by pretreatment with NBMPR-P (Kolassa et al., in press), suggesting that handling of nucleosides other than arabinosides may be unusual in the spleen.

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Appendix:

Distribution of i.v. administered [2,8-^{3}H]araAMP in tissues of the mouse. Female B10D2F$_1$ mice were injected i.v. with 15 mg/kg araAMP, and tissues were sampled for assay of ^{3}H content at the indicated times after drug administration. For all tissues, n = 5, data are expressed as means ± S.D.

	Tissue ^{3}H concentration (nmol/g)$^+$			
Tissue	7.5 min	15 min	30 min	60 min
Blood	31.8 ± 4.3	24.2 ± 2.3	14.5 ± 2.0	9.6 ± 2.7
Plasma	30.9 ± 4.1	23.6 ± 1.9	12.3 ± 1.3	5.6 ± 3.0
Brain	3.50 ± 0.66	3.84 ± 0.41	5.21 ± 1.13	4.34 ± 0.98
Mesentery	20.5 ± 4.6	15.5 ± 2.1	10.6 ± 2.2	15.5 ± 9.2
Pancreas	34.6 ± 4.3	28.1 ± 5.8	21.7 ± 2.7	26.5 ± 12.6
Heart	36.7 ± 6.4	27.3 ± 4.7	21.6 ± 5.4	13.6 ± 3.5
Lungs	33.0 ± 8.3	29.1 ± 4.1	24.5 ± 4.0	16.0 ± 3.0
Uterus	23.3 ± 6.4	26.1 ± 3.3	22.3 ± 6.7	14.6 ± 4.2
Muscle	26.6 ± 2.6	20.3 ± 3.2	13.9 ± 1.1	11.3 ± 3.9
Thymus	39.2 ± 8.6	32.2 ± 2.7	26.6 ± 3.9	13.4 ± 4.9
Spleen	126.1 ± 14.3	150.0 ± 20.1	138.1 ± 11.9	104.9 ± 28.6
Kidneys	68.5 ± 10.3	53.3 ± 6.8	40.3 ± 6.5	42.4 ± 7.7
Stomach	18.2 ± 5.1	18.2 ± 12.5	18.2 ± 15.1	29.1 ± 7.0

(... Appendix cont'd ...)

Tissue	Tissue ^{3}H concentration (nmol/g)$^{+}$			
	7.5 min	15 min	30 min	60 min
Jejunum	23.0 ± 4.6	23.0 ± 4.0	25.7 ± 8.1	27.3 ± 8.8
Ileum	25.5 ± 4.0	20.1 ± 3.5	20.8 ± 7.3	25.2 ± 8.7
large Int.	18.2 ± 3.4	16.3 ± 2.9	12.7 ± 2.5	15.6 ± 3.1
Liver	63.4 ± 8.8	57.3 ± 6.3	55.1 ± 5.3	57.6 ± 5.4